Ruminant physiology

Digestion, metabolism and impact of nutrition on gene expression, immunology and stress

edited by:
K. Sejrsen
T. Hvelplund
M.O. Nielsen

Ruminant physiology

Ruminant physiology

Digestion, metabolism and impact of nutrition on gene expression, immunology and stress

edited by:

K. Sejrsen

T. Hvelplund

M.O. Nielsen

Wageningen Academic
P u b l i s h e r s

Subject headings:
Cattle
Agriculture
Feed

ISBN: 978-90-76998-64-0
e-ISBN: 978-90-8686-566-6
DOI: 10.3920/978-90-8686-566-6

First published, 2006
Reprint, 2008

Foreword

The X International Symposium on Ruminant Physiology took place in Copenhagen, Denmark in early September 2004. The previous meetings were held in Nottingham, UK (1960), Ames, USA (1965), Cambridge, UK (1969), Sydney, Australia (1974), Clermont-Ferrand, France (1979), Banff, Canada (1984), Sendai, Japan (1989), Willingen, Germany (1994) and Pretoria, South Africa (1999). After careful consideration it was decided that the next meeting will be held in France in 2009.

As documented in the chapters published in this book, the programme and the format of the meeting followed the tradition of these meetings and the guiding principles set up by the guidance committee in Pretoria. The chapters cover a wide range of topics spanning from digestion and absorption over metabolism, reproduction and lactation. Progress in knowledge within important issues related to rumen fermentation, absorption mechanisms and splanchnic metabolism is treated in nine chapters. A number of chapters address the relationship between nutrition and gene expression illustrating the important progress in scientific knowledge that can be obtained by applying the molecular biology methods to the field. Several chapters address the impact of nutrition with immunology and stress – topics that are highly relevant in view of high focus on health and welfare of production animals. In line with the increased attention on the relationship between food and human health, the book contains 2 important chapters on this topic. The submitted short papers were published in a 725 page long special issue of Journal of Animal and Feed Science (vol. 13, supplement 1, 2004) available at the meeting.

The organising committee would like to express thanks to all the many that have helped in the process. Special thanks are due to the chairs and co-chairs of the sessions that have helped organising the sessions and in reviewing the chapters and all the short papers. Similarly, special thanks are due to the sponsors, including the Royal Veterinary and Agricultural University, the Danish Institute of Agricultural Sciences and The Danish Council for Agricultural and Veterinary Research.

This book contains the main papers presented at the Xth International Symposium on Ruminant Physiology. Proceedings from these symposia have over the years had a major influence on the science and teaching in animal science. Without doubt the chapters in this book live up to this fine tradition. The authors that have written the various chapters are among the very best in the world in their specific fields.

The organising committee

Mette Olaf Nielsen
Kris Sejrsen
Torben Hvelplund
Peder Nørgaard
Jørgen Madsen

Table of contents

Part IV: Lactation and reproduction physiology

Part V: Lactation and reproduction physiology

Part VI: Nutrition and immunology

Part VII: Nutrition and stress physiology

Part VIII: Human health aspects

Part I: Rumen fermentation

Characterisation and quantification of the microbial populations of the rumen

J.L. Firkins and Z. Yu
The Ohio State University, Department of Animal Sciences, 2029 Fyffe Road, Columbus, OH 43210, USA

Abstract

Already more than 3000 bacterial 16S rRNA gene sequences of rumen origin have been archived in public databases. Molecular-based approaches have been adapted more recently to protozoa and archaea, which also appear to be more diverse than once thought. Although providing a much more comprehensive description of bacterial diversity, sequencing approaches have limitations when extended to functional diversity and ecology. Quantification of important groups or species using molecular-based procedures can help explain their role in ruminal metabolism but also to provide perspective for more qualitative approaches. Herein, we have reviewed recent microbial ecology studies, mostly those using molecular biology approaches, with an emphasis on quantitative aspects and the relationship to ruminant nutrition. Advancing knowledge has led to more questions about how microbes interact in communities within different rumen compartments and even among animals fed similar diets. We suggest that future efforts should be directed toward the cultivation of the previously uncultured bacteria and the use of high throughput strategies to further delineate true diversity and function of rumen microbial populations under different dietary conditions. Ruminant animal production should become increasingly more efficient when rumen microbial ecology and nutrition studies are integrated, particularly if research efforts are coordinated among laboratories.

Introduction

Rumen microbial ecology has been extensively researched for over half a century, and many outstanding reviews have systematically described the characterised predominant species of bacteria, protozoa, and fungi. Bacteria were typically classified and aggregated into functional groups according to morphology and motility, growth factors required, substrates degraded, and products produced in pure culture (Hespell *et al*., 1997; Stewart *et al*., 1997; Dehority, 2003). Morphological classification of protozoal species and their known effects on bacterial populations and host nutrition have been well described (Williams and Coleman, 1992; Williams and Coleman, 1997; Dehority, 2003). The role of fungi has been associated with the colonization of refractory plant particles (Dehority, 2003). Despite the advancements made, though, more work is needed to better characterise the impact of diet and feeding method on these populations of microbes, how they interact together synergistically and antagonistically, and how they are influenced by host factors. Some glimpses of the community structure have been provided by coordinated scanning electron micrographs after feeding different types of diets (Akin and Barton, 1983), and sampling from different phases or locations of the rumen has helped to define the roles of groups of microbes in the reticulo-rumen (Cheng and McAllister, 1997; Dehority, 2003).

The majority of bacteria have not been cultured or described, only some protozoa can be cultured axenically, and fungi exhibit a complex growth cycle. Thus, the more recent application of cultivation-independent molecular techniques has provided more sensitive and accurate alternatives to directly examine the diversity and the microbial community structure on the basis of genotypes, instead of phenotypes (Whitford *et al*., 1998; Tajima *et al*., 1999). Various RNA- and DNA-based approaches have been used and are continuing to evolve to provide the tools to change and improve our perspectives about ruminal microbial communities in vivo. More recent molecular-based data have highlighted how complex microbial communities in the rumen can be in both composition and structure. Expanding knowledge of microbial diversity is highly relevant from an evolutionary standpoint, explaining the development of highly specialized microbes inhabiting the gut (Mackie *et al*., 2000; Zoetendal *et al*., 2004). Ecologically, greater diversity is often considered a positive attribute for more stable and resilient microbial communities (Hunter-Cevera, 1998; Zoetendal *et al*., 2004).

Ruminal microbial communities are composed of various distinct groups mostly specialized in polysaccharide hydrolysis and fermentation of resultant sugars, so populations of microbes among animals fed similar diets would be expected to be similar. In contrast, a major roadblock for integration of the ever-expanding knowledge in rumen microbiology and ruminant nutrition will be to explain variation in microbial ecosystems among animals (Weimer *et al*., 1999; Sylvester *et al*., 2005) fed the same diets. There is a serious need to address the pros and cons of emerging techniques so that they can be utilized to more comprehensively characterise ruminal populations of microbes to explain these differences and allow better prediction of dietary responses. In particular, methodological limitations (Zoetendal *et al*., 2004; Yu *et al*., 2006) and the need for hundreds of thousands of rRNA sequences for adequate characterisation of the true microbial diversity and community composition (Hughes *et al*., 2001; Larue *et al*., 2005) will need to be addressed to effectively utilize molecular-based procedures for quantitative purposes in ruminant nutrition studies.

The efficiency of microbial protein synthesis in the rumen has a profound effect on ruminant productivity (Stern *et al*., 1994). Yet, more progress is needed to integrate the physiological processes in the rumen to maximize this efficiency consistently. Methodological issues with regard to measurement of microbial protein synthesis (Firkins and Reynolds, 2005) and differences among experiments (Oldick *et al*., 1999) need to be resolved for improved application in ruminant production systems. For example, although microbes need energy and protein degradation products in synchrony (Cotta and Russell, 1997), research documenting the importance of this synchrony is equivocal (Firkins, 1996), perhaps because of the large amount of urea-N recycling from the blood into the rumen (Firkins and Reynolds, 2005). Similarly, excessive carbohydrate degradation or resultant low pH have a profound effect on microbial growth (Wells and Russell, 1996; Dehority, 2003). Although initial pH had a residual inhibition on fiber degradation in batch culture (Mouriño *et al*., 2001), when evaluated in continuous culture with continual feeding, pH had little apparent residual depression on fiber degradation (Calsamiglia *et al*., 2002), perhaps because of continual influx of new feed particles. Because in vivo pH is very difficult to predict with accuracy (Allen, 1997), more studies will be needed to resolve the complexities of microbial populations and how they change with varying carbohydrate supplementation strategies that alter a number of host factors such as urea recycling and salivary buffering.

Microbiological and nutritional perspectives typically have been compartmentalized and too often with completely separate objectives, yet there are various opportunities for coordination. For example, the particle-associated bacteria (PAB) are considered the most important group of bacteria mediating fibrolysis in the rumen but also are the hardest from which to obtain a truly representative sample, which is needed to accurately quantify bacterial N flows to the duodenum (Stern *et al*., 1994; Volden, 1999). With regard to quantification of bacterial N, can molecular-based procedures help verify that the PAB samples collected using various buffers truly represent the entire PAB population, including those that won't detach without significant lysis (Balcells *et al*., 1998)? Various species produce unique isomers of fatty acids (Minato *et al*., 1988). Although bacteria apparently change their lipid composition with different growth conditions (Moon and Anderson, 2001), 'signature lipids' labeled with radioisotopes might be used in future studies to link microbial populations to specific metabolic processes (Boschker *et al*., 1998; Polz *et al*., 2003). Specific bacterial groups produce small amounts of highly specific, potent fatty acid isomers (Harfoot and Hazlewood, 1997) that regulate the expression of certain genes controlling the host's metabolism (Peterson *et al*., 2003). As we move to adapt information regarding genetic diversity among microbial populations toward functional objectives (Krause *et al*., 2003), more opportunities become available to move molecular-based procedures from the test tube to the animal, particularly to improve the efficiency of utilization of nutrients or to modify the composition of animal products. Thus, objectives of microbial ecology can increasingly be integrated with applied nutritional objectives to address societal and economic issues.

Our current objectives are to integrate studies characterising microbial populations in the rumen from a nutritional perspective but also from the standpoints of microbial diversity. We will limit the scope to populations of protozoa, *Bacteria* (phylogenetic domain classification) excluding obligate amino acid fermenters, and *Archaea* (methanogenic prokaryotes) in the rumen. Whenever possible, we will integrate this information and provide considerations for potential future research.

Enumerating and characterising prokaryotic and protozoal populations by culture-based and microscopic methods

Microbiologists in the 1950's to 1980's worked out cultivation procedures to grow bacteria from the rumen (Dehority, 2003). Specific growth factors, products formed, and other landmark types of information have been gleaned using this approach. Although many types of cross-feeding and synergistic/antagonistic relationships have been identified (Wolin *et al*., 1997), most examples of community ecology really can't be mimicked using standard isolation procedures (Tajima *et al*., 1999). For example, proteinaceous substances are produced by some strains of bacteria to inhibit other strains (Chan and Dehority, 1999; Rychlik and Russell, 2002). Because these inhibitors seem rather ubiquitous in ruminal bacteria (Kalmokoff *et al*., 2003; Chen *et al*., 2004) but there could be differences among continents (Cookson *et al*., 2004), their impact on microbial ecology and its relationship to feed degradation and microbial N turnover in vivo is unclear. In the past decade or two, axenic culture of rumen protozoa has been refined (Dehority, 1998; Nsabimana *et al*., 2003). Still, many species of protozoa have yet to be grown successfully in axenic culture. As will be discussed later, cultivation of previously uncultured species of bacteria and protozoa is

needed to understand their function but also to provide a better foundation for molecular-based procedures to characterise microbial communities in vivo.

Using culture-based techniques, total bacterial numbers have been reported to decrease after feeding (Leedle *et al*., 1982); the authors suggested that the decrease could be directly associated with feeding (e.g., lysis associated with rising oxygen concentrations). However, dilution of bacterial counts in fluid by rapid adherence to new feed particles (Fields *et al*., 2000) or by dilution by drinking water are other likely explanations. When cattle were fed every 2 h to simulate a steady state, Firkins *et al.* (1987b) noted that increasing feed intake increased the total viable counts of bacteria in the rumen pool and the viable counts per gram of organic matter apparently degraded in the rumen. However, increased feed intake decreased almost by half the percentage of counts associated with ruminal fluid. The authors noted that protozoal counts were not related to feed intake, so protozoal predation appeared not to be a major factor. They speculated that increasing dilution rate of fluid and small particles passing with the fluid washed out fluid-associated bacteria (FAB). Grinding forages increases colonization by bacteria (Bowman and Firkins, 1993) but often increases their passage rate (Van Soest, 1994). Fibrolytic bacteria have intricate adhesion mechanisms that are critical for their degradative abilities (Miron *et al*., 2001), so particle passage rate would significantly impact PAB populations. More integrative objectives relating factors such as particle size and passage rate to microbial ecology are needed and can be facilitated with the increasing availability of molecular-based techniques. We note that sampling site, differences in microbial populations among animals, and other factors (Martin *et al*., 1999; Kocherginskaya *et al*., 2001; Michalet-Doreau *et al*., 2001; Koike *et al*., 2003a) will need to be considered in future studies.

Enumeration of bacterial species or groups based on selective media (Dehority, 2003) has several limitations, including 1) inability to account for viable but non-dividing cells of culturable species (Leedle *et al*., 1982), 2) colony-forming units that were actually initiated by clumps of cells (Dehority, 2003), 3) inability to grow some (or perhaps the majority of) species in laboratory media (Wells and Russell, 1996), and, although often overlooked, 4) the statistical variation inherent with cultivation-based procedures. Many authors have assumed that the difference between total direct counts and total viable counts of bacteria is largely attributed to nonculturable bacteria in the samples (Wells and Russell, 1996; Zoetendal *et al*., 2004). Dehority (2003) described potentially confounding factors among some studies e.g., not long enough incubation prior to growth scoring (colony counting). Both excessive and deficient amounts of substrate can increase lysis (Wells and Russell, 1996). In contrast, Krause *et al.* (1999b) argued that cellulolytic most-probable number (MPN) probably overestimates the truly cellulolytic population because of the presence of cellulodextrin degraders that cannot degrade polymeric cellulose. Similarities among some substrates and the ability of many bacteria to use multiple substrates also hinder quantitative characterisation of bacterial populations using cultivation-based approaches. For example, the presence of both cellulose and xylan in MPN may underestimate the fibrolytic population compared with when these substrates are used separately because many bacteria can use both substrates. Finally, MPN enumeration, while being much more amenable for use in animal experiments, had less precision than actual counting methods (Dehority *et al*., 1989) and can lead to inaccuracy in quantification of bacterial populations, depending on the type of MPN statistics used (McBride *et al*., 2003).

Protozoal enumeration in the rumen also is problematic. Sampling from the rumen is probably even more difficult than for bacteria because of the cyclic chemotaxis of some protozoa, especially isotrichids, toward sugars after the animal is fed, followed by settling to the ventral rumen or reticulum (Dehority, 2003) from which sites are harder to sample. However, many of these studies have been done with sheep, which, because of their much smaller rumen fistulas, might be much more difficult to collect representative samples from the entire rumen compared with cattle. Despite the standardization of counting procedures, variation in protozoal counts among animals is high such that differences among dietary treatments are hard to ascertain with statistical significance (Oldick and Firkins, 2000). Morphology-based speciation and enumeration is also very difficult. For example, Ito *et al.* (2001) distinguished six species of *Ostracodinium* using 44 diagrams in 21 figures, and this is a minor genus compared with *Entodinium*. Some protozoal species may simply be only morphological variants of the same operational taxonomic unit (Dehority, 1994). Sample dilution to yield a targeted number of cells might result in inaccurate and imprecise enumeration of minor species because the latter are often so diluted out that they are infrequently detected per microscopic field. Finally, the cell number to biomass ratio is quite variable in protozoa. The ranges in length and width within and among the *Entodinium* species evaluated were often larger than the means of those measurements (Dehority, 1994).

Although much useful information has been generated from co-cultures of bacteria (Wolin *et al*., 1997; Dehority, 2003), extrapolation of data to in vivo situations might not be appropriate. For polymeric cellulose as substrate, bacterial growth rate is probably dependent on the surface area for bacterial adhesion (Fields *et al*., 2000), which increases and then decreases after provision of substrate. If growth is assessed as the change in optical density of a culture, then the substrates should be soluble. For steady state growth on soluble substrates, growth rate (μ) depends on the maximum growth rate (μ_{max}), the substrate concentration (S) and the affinity toward that substrate (K_s): $\mu = \mu_{max} / [1 + (K_s/S)]$ (Dijkstra *et al*., 1998). Growth of adherent bacteria in planktonic conditions can skew competition toward those with higher affinity for soluble substrates. If substrate concentration is much higher than the K_s (the lower the value, the higher the affinity), then differences in K_s among species have a small effect on bacterial growth. However, as initial substrate concentration decreases to approach the lower K_s, then the K_s has a progressively more dramatic effect on subsequent bacterial growth. For batch cultures, the substrate would be declining over time, progressively benefiting the growth of the species with a lower K_s. For co-cultures in a steady state system, the concentration of substrate will decline from that in the infusion medium to a constant concentration in the culture vessel that is dependent on the growth rate and infusion rate. Similarly to batch cultures, as the substrate concentration decreases closer to the K_s, then the relative K_s of the two species will have an increasingly larger effect. Thus, bacteria grown in planktonic conditions lose advantages that they have developed for in vivo competitiveness (Shi *et al*., 1997), and the interpretation of competitive bacterial growth of individual species in co-cultures would depend on substrate concentration.

Cultivation-based studies of ruminal microbes have yielded useful information, especially for microbial physiology. However, only a small portion of the ruminal bacteria can be grown in laboratory media (Stewart *et al*., 1997). The inability to grow all ruminal microbes has therefore limited our understanding of ruminal microbial communities, such as diversity, community structure, population dynamics, and degradation and transformation of dietary components. Such

limitations of cultivation-based approaches have been partially overcome with the application of molecular biology techniques, advancing our knowledge about ruminal microbial communities considerably in the past two decades.

Characterising bacterial populations by molecular biology techniques

Roselló-Mora and Amann (2001) argued that "chemotaxonomy" should be used in conjunction with genomic information for a more complete "phylo-phenetic" characterisation of microbial populations. They have provided an extensive background, including a lengthy list of definitions of terms, on the history and strategy for molecular biology techniques used in microbiology. For example, although the large subunit (LSU) ribosomal RNA (rRNA) and the intergenic spacer region between the LSU and small subunit (SSU) rRNA probably contain more phylogenetic information than SSU rRNA, the latter received much more attention because of earlier sequencing limitations and subsequent accumulation of large numbers of SSU rRNA sequences deposited in public databases. The large amount of SSU rRNA or the corresponding gene (rDNA) sequence information continues to drive current research approaches. Although many procedures provide promise for characterising the populations of rumen microbes, particularly if used in combination with other emerging technologies (Polz *et al*., 2003; Zoetendal *et al*., 2004), cloning and random selection of clones for sequencing of SSU rRNA genes has been used more frequently to date to uncover the diversity of microbes in the rumen.

Dendrograms from rRNA or rDNA sequences generally provide relatively stable clusters, although the relative degree of branching depends on the method used (Rosselló-Mora and Amann, 2001). Goldman (1996) ranked methods used in the following order from most to least preferred: maximum likelihood, distance-matrix, neighbor-joining, and (not recommended) parsimony methods. However, for ecology studies, as opposed to those describing bacterial systematics, neighbor-joining seems to be widely accepted in microbiology fields, although maximum likelihood methods probably have more reasonable computing time nowadays. He cautioned that bootstrapping can infer higher confidence in branching estimates than is actually there.

We also noted a consistent trend in the literature in which a significant number of sequences have less than 90% sequence identity to those from characterised laboratory isolates, ranging from 56 (Tajima *et al*., 1999) to 42 (Koike *et al*., 2003b) to 25% (Larue *et al*., 2005). Even with the latter lower value, though, assuming a normal distribution, there are about as many sequences that have highest sequence identities that are quite distant as there are those close enough to known isolates to be classified in the same species. A species has been defined anywhere between 95 and 99% SSU rRNA sequence identity (Zoetendal *et al*., 2004). Rosselló and Amman (2001) discussed standardization of a species at 70% DNA similarity, which corresponds with approximately 97% rDNA similarity, although they cautioned that a species should not be distinguished solely on rRNA or rDNA sequence information.

An increasingly large amount of SSU sequence data are available in public databases to facilitate identification of rRNA sequences retrieved from rumen samples; the problem is that often many matched sequences are from uncultured bacteria, so renewed efforts to cultivate and characterise the 'uncultivable' bacteria would enhance our knowledge of the functional diversity of rumen

bacterial populations. Tajima *et al.* (2000) reported that almost all sequences derived from their study were >90% identical to those in the databases, but again many of these database sequences were derived from uncultured bacteria. Interestingly, they pointed out that a much larger percentage of their sequences were closely related to those from known laboratory cultures (especially numerous prevotellas) from cattle three days after a switch from a high-forage to a high-grain diet than either just before the switch or 28 days after the switch. The authors suggested that selection for self-sufficient bacteria that are opportunistic after a drastic diet change might mimic the same selective growth for bacteria isolated from the rumen, concluding that more cultivation studies are warranted. Comparing SSU rRNA sequences derived from the rumen, we found that a number of the same sequences (several examples are shown in Table 1) that were repeatedly retrieved from uncultured bacteria, but still we have no knowledge about the metabolism of these bacteria. Therefore, cultivation-based studies are needed to complement molecular biology techniques. Recent successful examples of cultivating 'not readily cultivable bacteria' in freshwater (Hahn *et al.*, 2004), seawater (Kaeberlein *et al.*, 2002), and soil (Stevenson *et al.*, 2004) habitats illustrate the types of procedures that could be combined with molecular analyses and directed toward rumen bacteria. Because the rumen is a defined microbial community of relatively specialized species and relatively limited diversity, a coordinated effort among research groups may lead to pure culture of most functionally important bacterial species.

Krause *et al.* (1999a) evaluated the diversity among *Ruminococcus* strains based on genotypic and phenotypic traits. All three genotyping procedures were needed to differentiate all 23 strains of *R. albus* and *R. flavefaciens*. Interestingly, based on the combination of the three genotyping methods used, three strains of *R. flavefaciens* clustered with the four *R. albus* strains rather than with the other *R. flavefaciens*. Four of the 23 strains significantly changed the grouping when the data were based on digestibility of cellulose. This study highlights another aspect for why characterisation of physiological properties of uncultured strains could complement phylogenetic placements to improve our ability to relate bacterial community structure to animal responses such as rumen fiber digestibility.

Table 1. Examples of repeated 16S rRNA sequences deposited in GenBank from uncultured rumen bacteria with 100 or 99% identity.[1]

Accession number	100%	99%	Closest known species (% identity)
AB034081	6	10	*Lactobacillus vitulinus* (95%); *Symbiobacterium* sp. (95%); *Bacillus* sp. (95%)
AY006929	0	8	*Clostridium aminophilum* (92%)
AY006928	4	14	*L. vitulinus* (94%)
AY006923	6	10	*L. vitulinus* (95%)
AY006920	4	14	*L. vitulinus* (94%)
AY006623	7	8	*Ruminococcus* sp. CO47 (90%); *R. bromii* (90%)
AY006647	0	18	R. sp. YE71 (90%)
AY006643	11	7	*Ruminococcus* sp. CO47 (88%)
AF293947	11	8	*Eubacterium* sp. oral clone (93%)

[1]Out of 2388 sequences of rumen origin at the time of accession.

Observations from cloning and sequencing studies for bacteria

As discussed previously, the majority of microbes in the rumen elude pure culture, and their existence is identified only from phylogenetic marker sequences (almost exclusively rRNA or its coding sequence, rDNA). In addition to constructing rDNA clone libraries from rumen fluid (Whitford *et al*., 1998), several researchers constructed rDNA clone libraries from fractionated rumen fluid and particle samples (Koike *et al*., 2003b; Larue *et al*., 2005). In these studies, random clones were selected for sequencing and phylogenetic analyses to examine the diversity and community composition. Selected data are reported in Table 2. In some of these studies, the data were reported directly; in others, we collated data from the published dendrograms. Data were grouped into phyla as described by Krause and Russell (1996).

Although *Bacteroidetes* (formerly termed *Cytophaga-Flexibacter-Bacteroides*) was suggested to be over-represented by cultivation-based procedures compared with sequencing data (Tajima *et al*., 2001a), the percentage of sequences belonging to this phylum ranged from 2 to 79% in Table 2. The major numeric competitor, *Firmicutes* (formerly termed Low G+C Gram-Positive Bacteria), ranged from 11 to 95%. When comparing data among studies, neither diet nor phase of the rumen contents seemed to be consistently related to relative abundance of *Firmicutes* versus *Bacteroidetes*. Differences among studies could be explained by the various PCR conditions (Tajima *et al*., 2001a), including choice of primers or cloning vector (von Wintzingerode *et al*., 1997), or from host factors that differ among experiments. Thus, broad conclusions regarding quantitative representation of various phyla are not possible. However, when compared within study, the *Firmicutes* appear to reside less in fluid than in particles; and the *Bacteroidetes*, more in fluid than in particles. Using specific oligonucleotides probes (data not shown), Krause *et al.* (2000) showed that the 16S rRNA from *Bacteroides-Porphyromonas-Prevotella* was generally about twice that of Gram-positive bacteria in ruminal fluid of lambs.

Diet influences the diversity and community composition within studies. Sequences clustering with *Treponema* (*Spirochaetes* phylum) were found only in cows fed a hay diet, and those clustering with *Ruminobacter amylophilus* (*Proteobacteria* phylum) were greatly increased in cows fed a grain-based diet (Kocherginskaya *et al*., 2001). Tajima *et al.* (2000) noted increased frequencies of sequences similar to known lactate-producing and lactate-utilizing bacteria three days after a shift to a high-grain diet. They suggested that the persistence of lactate utilizers after that transition might help keep *Streptococcus bovis* at low abundance. Similarly, although several sequences associated with cellulolytic ruminococci were detected prior to the diet shift, none were detected thereafter. Using the same primers and diets similar to the previously discussed high forage diet, the same group (Tajima *et al*., 1999) noted lower prevalence of *Firmicutes* in rumen fluid. Prevotellas were found fairly equally distributed between solid and liquid phases. However, only solids revealed *R. flavefaciens*, and only the fluid revealed *S. ruminantium* and *Proteobacteria*.

Koike *et al.* (2003b) evaluated cloned sequences obtained from forage particles incubated in situ. Because the nylon bags were rinsed stringently in water, firmly adherent fibrolytic bacteria should be enriched compared with FAB. This is one of the few studies that identified sequences related to *Fibrobacter succinogenes*, which, although considered a numerically important fibrolytic in

the rumen, rarely is represented in 16S rDNA clone libraries. Interestingly, sequences sharing high identity with *Treponema* were detected only on the alfalfa particles. The distribution of sequences recovered could be related to animal (one sheep per forage) or time of incubation (6 vs. 20 h) that varied among forages/sheep. Few sequences associated with ruminococci were identified, but numerous sequences associated with *Butyrivibrio fibrisolvens* were identified for both forages. Despite the pre-washing, there were numerous sequences clustering with the prevotellas, suggesting the involvement of these bacteria in the formation of biofilm adherent to digesta particles of plant biomass.

Larue *et al.* (2005) examined the bacteria in three fractions (fluid, loosely adherent, and firmly adherent) of rumen content taken from sheep fed diets containing either orchardgrass hay or hay plus 30% concentrate. Major bacterial populations found in the fluid and firmly adherent fractions are listed in Table 2. The authors chilled rumen contents for 2.5 h and used a detergent to detach firmly adherent bacteria. They noted a shift toward *Ruminococcus* sequences closely matching starch-degrading strains when grain was fed. In both fluid and particles, sequences most closely matching *Selenomonas* and *Prevotella* were numerically well represented. Many sequences clustered with *Clostridium* but with a low sequence identity, suggesting the presence of fibrolytic clostridia that have not been cultivated. This result also supports the need for novel cultivation approaches to better characterise rumen clostridia. Feeding only forage increased the number of sequences only distantly related to current isolates of the *Firmicutes*. Feeding grain tended to increase the number of sequences that were more closely related with current isolates of *Selenomonas* and *Prevotella*, but the opposite trend was noted for *Ruminococcus* and *Butyrivibrio*.

Some insight can be gained through comparisons of clarified rumen fluid (Whitford *et al*., 1998) and washed forage particles in situ (Koike *et al*., 2003b). In the former study, numerous sequences clustered with *P. ruminicola*, but many had similarities less than 94% (Whitford *et al*., 1998). When only adherent bacteria from washed particles were examined, many sequences were clustered with prevotellas other than *P. ruminicola* or *P. bryantii*. Similarly, relatively few sequences clustered with *B. fibrisolvens* in clarified rumen fluid, but numerous ones from washed particles clustered with this species. Even with thorough washing to remove non-adherent bacteria, few sequences clustered with ruminococci. Thus, processing method prior to DNA extraction has a profound effect on diversity results.

Ruminococcus albus and *R. flavefaciens* have been poorly represented in clone libraries, and *F. succinogenes* has been virtually devoid. Although *F. succinogenes* was detected using specific primers, no *F. succinogenes*-like sequence was identified in clone libraries constructed using universal primers (Larue *et al*., 2005). Because universal bacterial primers amplified the 16S rDNA sequence of *F. succinogenes* from pure cultures of this species consistently (Tajima *et al*., 1999; Larue *et al*., 2005), the absence of *F. succinogenes*-like sequences in clone libraries is not due to the lack of this species in the rumen sample or due to mismatches of primers. Table 3 was constructed based on data from that report plus unpublished data provided by those authors. *Fibrobacter succinogenes* had a higher threshold cycle from real-time polymerase chain reaction (PCR) than other bacteria. Of note is that *F. succinogenes* didn't even exceed background until

Table 2. Phylogenetic clustering of cloned 16S rDNA sequences from rumen samples.[1]

Host	Diet	Primers	Phase	n	%					Reference
					F	B	S	P	O	
5 cows	65% forage/ 35% concentrate	274f, 1492r	Fluid	53	11	79	0	8	2	(Whitford *et al.*, 1998)
4 steers	Alfalfa-grass hay	341f, 534r	Fluid	68	25	68	4	3	0	(Kocherginskaya *et al.*, 2001)
4 different steers	20% hay/ 80% concentrate		Fluid	67	15	58	0	27	0	
5 cows, pooled rumen samples	75% alfalfa hay/ 25% concentrate	27f, 1544r	Fluid	51	90	4	2	4	0	(Tajima *et al.*, 2000)
Same 5 cows	3 days after switch from 25% to 92% concentrate		Fluid	58	72	22	0	5	0	
Same 5 cows	28 days after switch to 92% concentrate diet		Fluid	41	95	2	0	0	2	
2 cows, pooled samples	80% alfalfa-timothy hay/ 20% concentrate	27f, 1544r	Fluid	41	49	39	2	5	5	(Tajima *et al.*, 1999)
Same 2 cows	80% alfalfa-timothy hay/ 20% concentrate		Particles	42	69	29	2	0	0	
1 sheep	Orchard grass hay	530f, 1392r	Orchard grass, 6-h in situ	48	42	54	0	2	2	(Koike *et al.*, 2003b)
1 different sheep	80% alfalfa hay/ 20% concentrate		Alfalfa, 20-h in situ	43	47	30	19	2	2	
2 sheep	Orchard grass hay	S926f, L189r (included the ribosomal intergenic spacer region)	Fluid	98	57	40	0	3	0	(Larue *et al.*, 2005)
			Particles	157	70	25	0	0	4	
2 different sheep	70% orchard grass hay/ 30% corn		Fluid	120	54	41	0	1	4	
			Particles	134	73	25	0	1	1	

[1]Data are from studies with 10-15 PCR cycles using the primers shown (based on *E.coli* numbering). Genomic DNA was extracted from fluid or particulate phases of the rumen. The data are percentages of sequences out of the total number (n) of sequences evaluated for the *Firmicutes* (F; previously termed low G+C Gram-positive phylum), *Bacteroidetes* (B; previously termed *Cytophaga-Flexibacter-Bacteroides* phylum), *Spirochaetes* (S), *Proteobacteria* (P), and other (O) phyla.

after the 15th cycle; recall that Table 2 was constructed from studies with 10 to 15 cycles because those authors tried to reduce PCR bias.

A standard curve for real-time PCR is prepared by serially diluting a known amount of rDNA copies (Sylvester *et al*., 2004). When added to a PCR reaction, each successive dilution requires a longer cycle to reach the threshold signal (fluorescence units above background units). The first cycle above this threshold is the threshold cycle. The PCR efficiency = $10^{(-1/slope)} - 1$ (Stratagene Mx3000P™ Real-time PCR System Instruction Manual, La Jolla, CA, USA). For varying PCR efficiency, the total number of copies at each successive cycle would be the original 1 proportion of template copies plus the new proportion (P) of copies that are produced. Briefly, it can be shown that $10^{(-1/slope)} = 1 + P$. Because P (which is PCR efficiency) is calculated from the slope, by definition, the lower the PCR efficiency, the more negative the slope of standard curve (threshold cycle vs. log rDNA copies). Furthermore, if there was a lower efficiency (more negative slope), then we would expect a more positive threshold cycle (an inverse relationship). In contrast, the relationship between threshold cycle and slope of the respective standard curves from real-time PCR in Table 3 was positive (slope = 0.211; data not shown) and not strongly supported by statistics ($r^2 = 0.25$). The threshold cycle in regular samples (as opposed to standard curves) is affected both by initial conditions that influence amplification of starting templates (e.g., copy number per cell or molecules associating with DNA) and by amplification efficiency (P) of new copies, which increasingly predominate in PCR product produced from previous PCR product. However, the slope is determined only from later PCR cycles and therefore by the amplification efficiency of successive PCR products produced almost exclusively from PCR products. Thus,

Table 3. Threshold cycles and slopes of standard curves from various ruminal bacterial species using real-time PCR[a].

Species	Threshold cycle	Slope
Streptococcus bovis	6.736	-7.485
Selenomonas ruminantium	8.375	-6.322
Anaerovibrio lipolytica	8.412	-4.092
Prevotella bryantii	8.758	-5.585
Ruminococcus flavefaciens	8.821	-5.561
Treponema bryantii	9.071	-4.932
Prevotella albensis	9.592	-6.601
Eubacterium ruminantium	10.28[b]	-5.287
Prevotella ruminicola	10.98	-4.180
Succinovibrio dextrinosolvens	12.59	-6.313
Ruminobacter amylophilus	13.39	-4.915
Fibrobacter succinogenes	15.85	-4.040

[a]Genomic DNA template (30 ng each) from representative strains of pure cultures from each species was used under the same cycling conditions using the universal *Bacteria* primers 27f and 1525r (Tajima *et al*., 2001a). Slopes from the real-time PCR standard curve (threshold cycle vs. log of rDNA copies) were provided as personal communication by the authors.
[b]Poor extension or annealing were noted as potential problems (Tajima *et al*., 2001a).

the similar shapes of the amplification curves of Tajima *et al.* (2001a) and the lack of relationship between threshold cycle and slope in Table 3 support the probability that the under-representation of *F. succinogenes*-like sequences (in the 'other' category in Table 2) or perhaps *R. amylophilus* (*Proteobacteria* in Table 2) in clone libraries resulted from poor amplification from its genomic DNA during the early amplification cycles. The 16S rDNA and/or the DNA flanking the 16S rDNA of these bacteria likely interferes with the amplification of the 16S rDNA by forming inhibitory secondary structures.

Quantitative issues influencing the interpretation of bacterial clone libraries

Quantitative assessment of the effects of PCR efficiency has received minor attention for ruminal microbes compared with its potential importance. Using a predetermined mixture of three pure cultures, Polz and Cavanaugh (1998) noted that even progressing from 5 to 10 PCR cycles increasingly biased the frequency of PCR product relative to initial template. They suggested that pooling of PCR replications might provide enough PCR product from only 5 cycles while also reducing variation associated with replicate amplifications. Although conceptually valid, 5 cycles would only amplify a starting template to at most 16 copies of the PCR product, compared with 512 for 10 cycles; thus, a minimum number of cycles might be needed to dilute background genomic DNA (and associated compounds).

Logarithmic bacterial growth can be assessed mathematically as 2^x, where x represents the number of division cycles (Dehority, 1998). Similarly, assuming a constant efficiency of amplification of DNA templates, the product accumulation can be estimated using the formula $(1 + P)^{n-1}$, where 1 represents the proportion of initial copies and P is the fractional amplification efficiency (proportion of new copies) of each cycle (n) except for the first cycle (only the original copy of genomic DNA is retained, so n-1 for PCR product copies). In reality, PCR product accumulation decreases and then ceases during later cycles when the reagents become limiting (Polz and Cavanaugh, 1998). With this qualification, after 10 cycles, a single copy of genomic DNA would be amplified to $(1 + P)^9 = 512$, 322, 198, or 119 copies of PCR product for 100, 90, 80, and 70% efficiency, respectively. It follows that an original rDNA template comprising 5.1% of the community DNA would be reduced from 5.1 to 3.2, 2.0 and 1.2%, respectively, of its theoretical contribution after 10 PCR cycles. If 100% is standardized to the average efficiency, for a bacterial population of 1 or 2% of a community, which occurs regularly for functionally important populations, with only 100 clones sequenced, the resulting recovery of a single sequence representing that species would statistically be unlikely if its PCR efficiency decreased by more than 10% with respect to the average of the community. This point is accentuated when considering rDNA templates that are amplified with efficiencies that are greater than the average. Although overly simplified, this exercise clearly demonstrates that reduction of PCR cycles to 10 will decrease ramifications of PCR bias (Whitford *et al.*, 1998; Zoetendal *et al.*, 2004), but the bias still is potentially significant, especially when low numbers of clones are sequenced. The degree of error imposed by PCR bias in rumen bacterial communities is not known. However, it is clear that greater numbers of clones need to be sequenced to accurately assess microbial diversity and community compositions in the rumen, or novel combined approaches are needed to characterise the less abundant but still ecologically important bacteria in communities, which would otherwise not be represented by clone libraries (Holben *et al.*, 2004; Yu *et al.*, 2006).

Tajima *et al.* (1999) addressed issues such as low copy number of 16S rDNA per cell, PCR efficiency, cloning efficiency, and DNA extraction efficiency. Tajima *et al.* (2001a) reported that *F. succinogenes* probably has three copies of rDNA, and they suggested that *S. bovis* had only one rDNA copy. Thus, copy number difference did not explain why *F. succinogenes* was devoid in their clone library. For most bacteria (heavily weighted toward pathogens), the copy number ranges from 1 to 12 per cell (von Wintzingerode *et al.*, 1997), averaging 3.8 with a standard deviation of 2.9 (Fogel *et al.*, 1999). Conceivably, when the number of clones sequenced per study is small, the copy number difference could have a profound effect on the relative number of sequences discerned (Crosby and Criddle, 2003) and, thus, interpretations of bacterial diversity. Theoretically, the abundance of a specific bacterium could be estimated when considering its copy number relative to that in the weighted average of the community (Fogel *et al.*, 1999). However, experimental derivation of data, rather than use of theoretical calculations, would be needed for accuracy. In one recent study, various strains of *Bifidobacterium* of human origin were found to have from 1 to 5 copies of the rDNA operon per cell (Candela *et al.*, 2004). If initial growth rate after introduction of substrate of various types of bacteria is positively correlated with copy number (Fogel *et al.*, 1999), then feeding readily available substrate to a ruminant might bias phylogenetic-based characterisations toward rapidly growing starch and sugar utilizers with more rDNA copies.

Numerous authors cited herein have commented about the high percentage of bacterial phylotypes that were not closely related to any known species. However, few have commented about the bias introduced by PCR and cloning (Tajima *et al.*, 1999). In fact, this bias might be highly correlated with cultivation bias, if bacteria more tolerant of high substrate concentrations also possess higher 16S rDNA copy numbers for rapid growth following intermittent feeding of grain (Tajima *et al.*, 2000).

Kocherginskaya *et al.* (2001) hypothesized *a priori* that diversity would be greater for high forage than for mixed grain/forage diets, although subsequent diversity indices did not support this hypothesis in their study. In contrast, with higher number of cloned sequences using ribosomal intergenic spacer analysis, diversity tended to be greater for hay-only diets (Larue *et al.*, 2005). Despite the established importance of only a few main bacteria in fiber degradation (Krause *et al.*, 2003), some secondary colonizers apparently use degradation products from primary colonizers as substrate (Wells *et al.*, 1995). At present, information is lacking to truly ascertain if diversity is higher or lower with increasing forage because of an inadequate number of sequences identified in each sample. Larue *et al.* (2005) addressed the statistical ramifications more fully than many previous authors by increasing number of clones but also by estimating the numbers of clones needed to reach 50% and 90%, respectively, of actual diversity.

Assuming several hundred bacterial species, with the most abundant species being 10^9 cells and the least being 10^4 cells per gram of digesta, 50,000 random clones would have to be sequenced from clone libraries in order to have a 50% probability to sequence that least abundant species (N.R. St-Pierre, personal communication); this assumes no potential PCR or cloning bias, which would further increase the number. However, due to labor and cost limitations, even the most ambitious efforts only sequenced several hundreds of clones (Larue *et al.*, 2005). Thus,

comprehensive examination of microbial diversity and community composition in the rumen requires more efficient high throughput sequencing approaches.

Recently, Neufeld *et al.* (2004) reported an innovative approach, termed serial analysis of ribosomal sequence tags (SARST), which permits efficient sequencing of thousands or more 16S rDNA sequences in single experiments. SARST uses a series of enzymatic reactions to amplify and ligate ribosomal sequence tags (RSTs) from SSU rDNA V1-regions, the most hyper-variable region (Yu and Morrison, 2004a), into concatemers that are subsequently cloned and sequenced. This approach offers a significant increase in throughput over traditional SSU rDNA clone libraries; up to 20 RSTs can be obtained from each sequencing reaction. Although SARST increased efficiency and throughput capacity, it requires numerous lengthy procedures and 7 to 8 working days to complete. More recently, Yu *et al.* (2006) developed an improved version of SARST (referred to as SARST-v1) by designing new primers. The SARST-v1 method substantially streamlined and simplified the SARST procedures, while maintaining the efficiency and high-throughput capacity. In a test of the SARST-v1 procedure using DNA prepared from the adherent fraction previously used by Larue *et al.* (2005), the authors identified, by sequencing a small portion of an SARST-v1 library, more than 250 unique RSTs (97% sequence similarity cutoff), which provides species-level resolution. The RSTs identified through SARST-v1 fell into 9 phyla. Within the *Firmicutes*, 30 genera were identified. The authors maintained that the RSTs identified through SARST-v1 can be used to design microarrays, which are more powerful for large-scale comparative analyses of microbial communities. Although needing further verification for applications to the rumen, such comprehensive analysis of microbial community composition should considerably further our knowledge on the functional implications of diversity and ecology of bacterial communities.

An often neglected issue in studies employing molecular biology techniques is the differential efficiency of RNA or DNA recovery from community samples. Differences in bacterial cell wall structures can lead to differential cell lysis of bacteria (Moré *et al.*, 1994), biasing results (von Wintzingerode *et al.*, 1997). Recently, Yu and Morrison (2004b) reported a new DNA extraction method that offers significantly improved DNA yields (up to 6 fold) over other DNA extraction methods. Conceivably, such improvement should be directly translated into more efficient cell lysis and representative community DNA.

The ability to study microbial diversity and community structure in vivo or in situ using molecular biology techniques offers large potential advantages in its quantitative description that is more representative of the community's actual ecology. However, the reader needs to be cautioned that molecular procedures can have high precision (repeatability) that could mask low accuracy (Polz and Cavanaugh, 1998) and even overestimate the real diversity in microbial populations (Polz *et al.*, 2003).

Quantification of microbial populations in the rumen

Relatively soon after the development of rRNA-based procedures, researchers adapted them for quantitative purposes in a variety of ecosystems. One large impetus for these objectives is that the procedures should be able to quantify bacteria that are not readily culturable and are therefore undercounted (Krause *et al.*, 1999b). Many quantitative issues affecting the accuracy

and precision of these techniques were described (Raskin *et al*., 1997), and reported variation was relatively large (Weimer *et al*., 1999; Krause *et al*., 1999b). Quantitative extraction of RNA is difficult (Krause *et al*., 1999b) and variable, depending on its concentration relative to other carrier biomass (Raskin *et al*., 1997). Krause *et al.* (2000) estimated that *Bacteria* rRNA was about 60 to 70% of the total rRNA signal. The total *Eukarya* signal was less than 10%. *Archaea* signal was not measured directly in that study, but, by difference, its signal would be about 25%, which is much higher than the *Archaea* signal directly quantified in ruminal contents (Ziemer *et al*., 2000) or even expected from microscopic count data (Krause *et al*., 1999b). The rRNA in all studies was normalized against total SSU rRNA (including archaeal, fungal, protozoal, and perhaps even plant and bovine) to help overcome methodological inconsistencies such as variation among nylon membranes. However, a recent report (Hocquette and Brandstetter, 2002) cautioned that the slope of signal intensity with increasing quantities of target might be different than the slope from the standard, so normalization using single values might not be valid unless common slopes are verified. There is profound variation in ribosome numbers/cell over a growth cycle (Raskin *et al*., 1997; Zoetendal *et al*., 2004), but few studies quantifying rRNA addressed this issue. Even if total rRNA is quantitatively determined (Ziemer *et al*., 2000), the variable rRNA concentration per cell requires conversion to a per cell basis or per unit of cellular N. Thus, the rRNA hybridization data should be considered to be semi-quantitative and useful only for making general comparisons.

Such problems with rRNA-based procedures prompted the development of quantitative methods targeting rDNA. Competitive PCR allows for a competing standard to be used as would an internal standard (Reilly *et al*., 2002). Thus, assuming that any PCR inhibition would equally affect both the target DNA and the competitor DNA templates, the quantification should be accurate (Fogel *et al*., 1999). However, competitive PCR is time-consuming and thus simply much less feasible for routine usage in studies involving a large number of samples, elevating real-time PCR to be the current method of choice for many researchers (Schmittgen *et al*., 2000; Ouwerkerk *et al*., 2002b).

When using both real-time (Ouwerkerk *et al*., 2002b; Klieve *et al*., 2003) and competitive (Koike and Kobayashi, 2001) PCR, serial spiking of known numbers of pure cultures into ruminal fluid consistently resulted in linear recoveries. The abundance of target populations present in the unspiked rumen fluid was also calculated based on the intercept differences in standard curves between spiked and unspiked samples (Ouwerkerk *et al*., 2002a; Ouwerkerk *et al*., 2002b) or using non-linear regression after spiking serial dilutions of a standard into a constant amount of the sample (Sylvester *et al*., 2004). Such a sample-derived standard should be a weighted mean of all populations in the microbial community. This standard from each sample could have DNA quantitatively extracted for 1) determination of rDNA copies and 2) generation of PCR product. Because the standard curve in real-time PCR should have the same mix of rDNA as the sample rDNA, both should theoretically amplify with the same efficiency. The harvested microbial standard also could be used to quantify N concentration, so total rDNA copies can be used as a microbial marker to quantify the amount of N. Details have been described systematically for determination of protozoal N (Sylvester *et al*., 2004).

Specificity of probes or primers to their targets is prerequisite for accurate quantification of any populations of microbes. Specificity can be designated to individual strains, species, groups within a genus, or genera. Several researchers have quantified bacteria in the rumen, and selected data are presented in Table 4. In the study of Krause *et al.* (1999b), the total combined signal from *R. albus*, *R. flavefaciens*, and *F. succinogenes* was 4.0% of the total bacterial 16S rRNA. These data were in general agreement with total cellulolytic counts (5.2% of total culturable counts or 3.1% of direct counts).

In the study of Michalet-Doreau *et al.* (2001), *F. succinogenes* and *R. flavefaciens* were more enriched in PAB and firmly adherent PAB populations when compared with the total bacteria (which included FAB), whereas rRNA from *R. albus* was comparable in all phases (Table 4). Based on these observations, one would expect FAB to be less enriched with fibrolytics than would the PAB, again helping to explain the under-representation of these microbes, particularly *R. flavefaciens*, in many clone libraries prepared from rumen fluid. Compared with other studies reported, Weimer *et al.* (1999) determined low rRNA abundance of *F. succinogenes* and *R. flavefaciens* relative to *R. albus* and to results from other studies. However, when considering all studies using rRNA probes in Table 4, the relatively high concentration of rRNA from *F. succinogenes* and the *Ruminococcus* species supports our previous assertions regarding under-representation of these microbes in clone libraries.

In general, the studies listed in Table 4 show relatively higher percentages of rRNA from these cellulolytic species with all-forage than with mixed diets containing grain, as would be expected, but there have been few direct comparisons within a study. In the study of Weimer *et al.* (1999), diet had no statistically significant impact ($P > 0.23$) on rRNA percentages of the three cellulolytic species, but differences among cows ($P = 0.04$) were detected for *R. flavefaciens*. Host specificity has been well described for gut bacterial communities (Zoetendal *et al.*, 2004). When using competitive PCR, no significant differences were detected in abundances of these three species (Koike and Kobayashi, 2001) among treatments ranging from 80 to 20% forage. The low estimated cell numbers or lack of differences in diets could be a result of their using a single sheep; changes in total cellulolytic bacteria might be more pronounced if a larger number of animals had been used.

In a study designed to characterise changes in populations of proteolytic bacteria as affected by varying supplementation rate of N on pasture with or without a combination of dextrose and corn flour (Reilly *et al.*, 2002), DNA was extracted from 84 strains of different species to verify primer specificity. The authors noted an apparent opposing trend of increasing some strains with increasing carbohydrate that corresponded with decreasing detection of *Eubacterium*. In addition, they noted that *Streptococcus* species were lower when carbohydrate was fed but only with pastures with lower protein, whereas *P. bryantii* was higher in that diet. The quantification of the total *Prevotella* species remained high and constant across diets. Although the authors acknowledged that the accuracy of data for total bacteria is not known because many strains have not been sequenced, their estimate of total bacterial cell numbers remained constant at 1.6 to 2.0 x 10^{10} cells/ml, which approximates expected numbers based on counting methods.

Table 4. Selected results from studies quantifying ruminal bacteria using molecular techniques[1].

Host/Diet	Rumen phase	Procedure	Target bacteria	Results	Reference
3 steers fed orchardgrass hay at maintenance	FAB from fluid FAB from mat	rRNA probe	*Fibrobacter succinogenes* S85	5.6% of total 16S rRNA 6.4% of total 16S rRNA	(Briesacher *et al*., 1992)
1 cow fed 60% alfalfa hay and 40% concentrate	Total rumen content	rRNA probe	*Fibrobacter* genus *F. succinogenes* *F. succinogenes* subgroup 1 *F. succinogenes* subgroup 3	2.2% of total 16S rRNA 2.0% of total 16S rRNA 0.62% of total 16S rRNA 0.56% of total 16S rRNA	(Ziemer *et al*., 2000)
3 sheep fed Rhodes hay and protein, every 3 hours	Total rumen content	rRNA probe	*F. succinogenes* *Ruminococcus flavefaciens + albus*	1.8% of bacterial 16S rRNA 2.2% of bacterial 16S rRNA	(Krause *et al*., 1999b)
15 lambs fed alfalfa hay and milk or milk replacer	FAB from stomach tube	rRNA probe	*F. succinogenes* *R. flavefaciens + albus*	2 to 4% of bacterial 16S rRNA 3 to 5% of bacterial 16S rRNA	(Krause *et al*., 2000)
4 dairy cows fed mixed diets varying in alfalfa and corn silage levels	PAB extracted by chilling	rRNA probe	*F. succinogenes* *R. flavefaciens* *R. albus*	0.22% of bacterial 16S rRNA 0.16% of bacterial 16S rRNA 1.1% of bacterial 16S rRNA	(Weimer *et al*., 1999)
4 sheep fed 40% alfalfa hay/60% barley concentrate	PAB by filtration and washing	rRNA probe	*F. succinogenes* *R. flavefaciens* *R. albus*	2.1% of bacterial 16S rRNA 1.4% of bacterial 16S rRNA 1.1% of bacterial 16S rRNA	(Michalet-Doreau *et al*., 2002)
4 sheep fed alfalfa hay	Total bacteria	rRNA probe	*F. succinogenes* *R. flavefaciens* *R. albus*	4.3% of bacterial 16S rRNA 1.6% of bacterial 16S rRNA 0.81% of bacterial 16S rRNA	(Michalet-Doreau *et al*., 2001)
4 sheep fed alfalfa hay	PAB	rRNA probe	*F. succinogenes* *R. flavefaciens* *R. albus*	5.8 % of bacterial 16S rRNA 2.5% of bacterial 16S rRNA 0.93% of bacterial 16S rRNA	
4 sheep fed alfalfa hay	Firmly adherent PAB by screening and washing	rRNA probe	*F. succinogenes* *R. flavefaciens* *R. albus*	6.3% of bacterial 16S rRNA 2.7% of bacterial 16S rRNA 0.73% of bacterial 16S rRNA	
1 sheep fed 80/20, 50/50, and 20/80 alfalfa hay/ concentrate diets	Total bacteria	Competitive PCR	*F. succinogenes* *R. flavefaciens* *R. albus*	0.6 to 4.5 x 10^7cells/ml 3 to 6 x 10^5 cells/ml 3 to 5 x 10^4 cells/ml	(Koike and Kobayashi, 2001)
8 dairy cows fed fresh pasture ± dextrose and cornstarch	All bacteria	Competitive PCR	Some *Butyrivibrio fibrisolvens* *Streptococcus* genus *Prevotella* genus *P. bryantii* Total bacteria	~1.1 x 10^8 cells/ml ~1.3 x 10^8 cells/ml ~1.3 x 10^9 cells/ml ~1.2 x 10^7cells/ml ~1.8 x 10^{10} cells/ml	(Reilly *et al*., 2002)
10 steers fed Paspalum hay and gradually switched to 75% rolled barley	FAB	Real time PCR	*B. fibrisolvens* *S. bovis* *Megasphaera elsdenii*	10^7 to 10^4 cells/ml 10^8 to 10^7 cells/ml 10^4 to 10^8 cells/ml	(Klieve *et al*., 2003)

[1]Bacterial samples were collected from fluid-associated bacteria (FAB) or particle-associated bacteria (PAB).

Klieve *et al.* (2003) demonstrated the potential for quantification of targeted bacterial rDNA to evaluate bacterial population changes as the diet was switched from high forage to high grain. *Butyrivibrio fibrisolvens*, noted for its ability to thrive under a variety of dietary conditions, decreased from about 10^7 cells/ml to nearly below detection limits after the dietary change. However, despite this pronounced effect, the *S. bovis* population remained stable, perhaps because of the dramatic increase in *Megasphaera elsdenii* (increased from about 10^4 to 10^8 cells/ml), a noted lactate utilizer. In the future, these types of objectives should be coupled with concomitant evaluation of enzymatic activity or other independent measures for accurate extrapolation of results from microbial community analysis for nutritional purposes. Lactate utilization was found to be of no taxonomic value for *Selenomonas ruminantium*, a major lactate utilizer with high diversity among strains (Zatkovic *et al*., 2000). Induction of synthesis of saccharolytic enzymes after feeding probably explained the increased degradative activity more than could be explained by the increased bacterial cell numbers (Williams *et al*., 1989). Systematic integration of genotypic with phenotypic measures will undoubtedly help account for microbial community differences among animals and(or) diets in future studies.

Quantification of protozoal biomass has been hampered by a suitable chemical marker (Firkins *et al*., 1998). To address this need for nutrition experiments studying microbial protein recycling in the rumen, Sylvester *et al.* (2004) developed a real-time PCR assay to quantify protozoal biomass in the rumen and duodenum. They optimized conditions and documented how results were influenced by the number of freezing/thawing cycles, DNA recovery after extraction and purification procedures, generation of appropriate rDNA standards representative of the sample to be analyzed, and statistical procedures for evaluating and corroborating recovery of target rDNA and numbers of replications. Acceptable accuracy and precision in the assay were documented. Using PCR-DGGE (denaturing gradient gel electrophoresis), we also found similar protozoal community profiles between the rumen and duodenum within animals (Sylvester *et al*., 2005), thus concluding that standards derived from the rumen are appropriate to quantify protozoal rDNA copies in the duodenum. This type of quantitative PCR assay should help researchers derive or improve mechanistic models integrating protozoal ecology and flow of microbial protein to the small intestine.

Protozoal ecology

The roles of various genera of protozoa and the benefits and detriments of ruminal protozoa have been well described (Williams and Coleman, 1992; Dehority, 2003). Although they contribute to fiber degradation and probably help slow the rate of fermentation by engulfing starch granules (Williams and Coleman, 1997), protozoa probably are the main antagonists for efficient usage of protein in the rumen microbial ecosystem (Wells and Russell, 1996; Wallace *et al*., 1997). Protozoa engulf large numbers of bacteria as their main nitrogen source (Williams and Coleman, 1997) or perhaps for other stimulatory reasons as yet undiscovered (Fondevila and Dehority, 2001). Up to half of the consumed bacterial N is excreted by protozoa (Jouany, 1996), and predation (termed 'grazing' by protozoologists) on bacteria decreases the economy of N usage in the rumen. Nitrogen recycling in the rumen has a major negative impact on whole-body N recycling (Lapierre and Lobley, 2001); however, defaunation has had relatively minor impact on blood urea N recycling

(Koenig *et al*., 2000) or N retention (Jouany, 1996), probably because dietary protein has been considerably higher than the animal's requirements in most experiments.

Besides decreasing bacterial protein outflow to the duodenum (Jouany, 1996), protozoa have been regarded by some to be virtually completely recycled within the rumen. This dogma was first established several decades ago based on the observation of much lower protozoa counts in the omasum than in the rumen. However, if the rumen was in a true steady state, ruminal lysis would not explain the lower counts in the omasum (counts would be equivalent). Lower omasal counts could be explained by protozoal lysis within the omasum (Michalowski *et al*., 1986). However, Towne and Nagaraja (1990) noted, in the omasum of 54 cattle and 40 sheep, rare evidence of disintegrating protozoal cells but sustained appearance of apparently viable protozoa.

Few authors seem to have commented that factors other than protozoal lysis can contribute to decreased protozoal counts in the omasum. Drinking water can pass from the rumen without mixing (Weller and Pilgrim, 1974; Cafe and Poppi, 1994), diluting the protozoal concentration in the omasum. Also, Firkins *et al.* (1998) expounded on how passage of ingested feed particles likely undergoes a delay in passage, which is followed by more rapid passage after the particles become less buoyant. Although the isotrichids (*Isotricha* and *Dasytricha* genera) are often discussed for their chemotaxis to sugars (Dehority, 2003), the entodiniomorphids also exhibit chemotaxis (Williams and Coleman, 1992). Right after eating, ruminal passage of feed is slow (Mathison *et al*., 1995). If protozoa are chemotactically attracted to solutes released from ingested feed, the passage of which is delayed, then protozoal passage should also be delayed. Thus, a simple ratio of protozoal counts in the omasum relative to those in the rumen should not be expected to represent ruminal lysis rates of protozoa. A more correct comparison of protozoal passage was done by Weller and Pilgrim (1974) and Michalowski *et al.* (1986). Similarly, when protozoal passage was estimated based on rumen fluid volume and dilution rate, the number of protozoa passing to the duodenum (volume x protozoal counts x dilution rate) ranged from 66 to 177% of their number in the rumen pool (Michalowski, 1998). However, using rumen fluid outflow appears to overestimate protozoal outflow because protozoa should not stay completely mixed with the ruminal fluid (Sylvester *et al*., 2005). In that study, protozoal N, calculated as a percentage of microbial (bacterial + protozoal) N, was similar in the rumen compared with the duodenum. Unfortunately, few other researchers have quantified omasal flow of protozoal cells relative to their pool size in the rumen from evacuated rumen contents, which should be the more correct way to assess net in vivo generation times of individual species using protozoal counts.

The concept of nearly complete lysis of protozoa in the rumen has been reinforced by numerous studies measuring duodenal flow of microbial N. As discussed previously (Firkins *et al*., 1998; Koenig *et al*., 2000), most marker methods (primarily quantifying protozoal N flow as the difference between total microbial N and bacterial N) used in previous experiments likely underestimate protozoal N because the procedural errors tend to underestimate total microbial N while overestimating bacterial N.

Isotrichids tend to settle to the bottom of the reticulum or ventral rumen, perhaps to scavenge more oxygen for more efficient ATP synthesis from degraded carbohydrate (Williams and Coleman, 1997). Although many authors have discussed isotrichids increasing in forage diets

because of their preference for sugars, Martin *et al.* (1999) showed that the numbers of isotrichids actually remained fairly constant (Figure 1) while the entodiniomorphids increased drastically when concentrate was added to the diet. These data show how expressing isotrichid counts as a percentage of the total counts could be misleading to their overall total numbers and ecology. More importantly, these data document the importance of time after feeding and sampling location to accurately represent protozoal populations. Based on the important interactions of protozoa with bacterial and archaeal prokaryotes, it follows that time after feeding and sampling location should impact our ability to assess those populations, also.

There has been extensive reiteration of earlier observations of proposed sequestration of isotrichid protozoa (Leng and Nolan, 1984; Dehority, 2003), yet the consistent similarity in generic distributions of protozoa in the rumen versus omasum (Towne and Nagaraja, 1990; Punia and

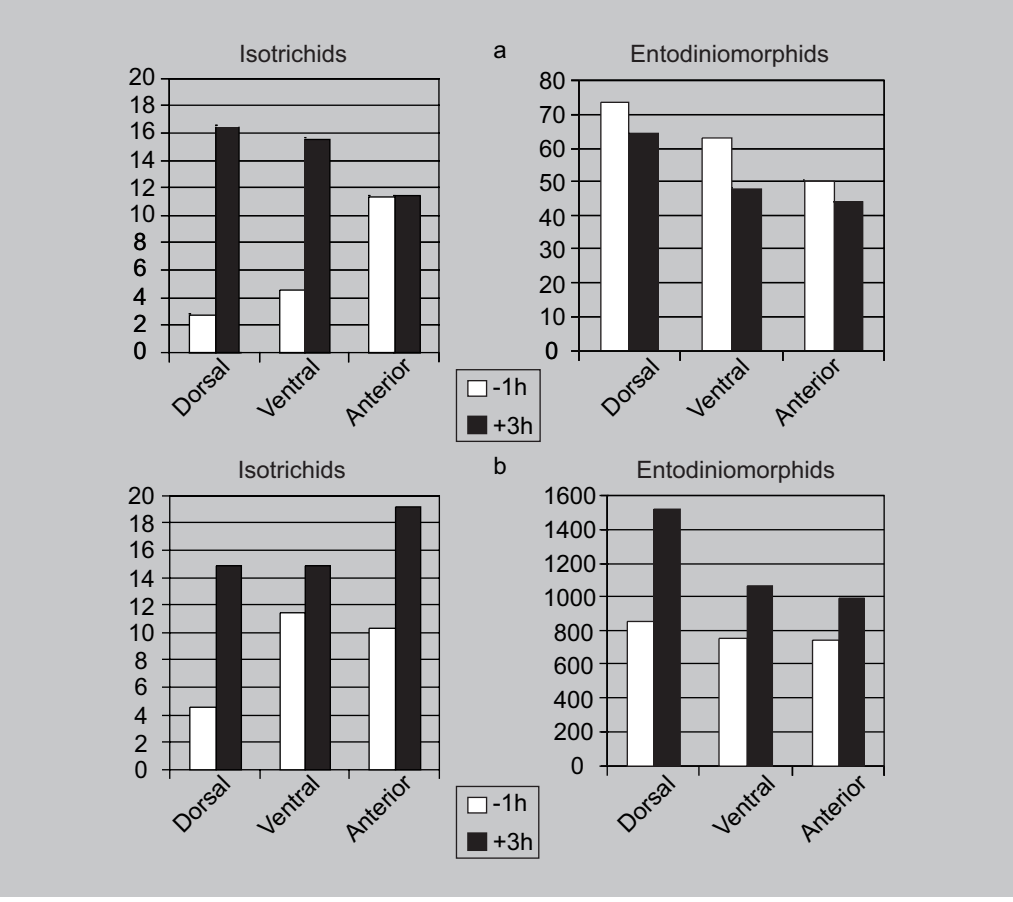

Figure 1. The effects of sampling time with respect to feeding either a high forage (panel a) or a mixed forage-grain diet (panel b) on populations of isotrichid or entodiniomorphid protozoa (cells x 1000/ml) in the dorsal, ventral, or anterior sacs of the rumen of cattle (Source: Martin et al.*, 1999).*

Leibholz, 1994; Michalowski, 1998) does not corroborate these expectations of differentially high lysis rates of isotrichids in the reticulo-rumen or, alternatively, that entodiniomorphids autolyse much more readily in the omasum than do isotrichids.

Although there has been in vitro evidence for the autolytic bursting of entodiniomorphids after uncontrolled consumption of substrate (Dehority, 2003), there has been little in vivo verification of such responses, probably because of technical limitations. Using screens to retain protozoa inside a container in the rumen of steers, Ankrah *et al.* (1990) suggested that isotrichids were less prone to lysis after eating than were entodiniomorphids; using certain assumptions, they calculated that lysis and passage contributed about equally to the loss of entodiniomorphids after feeding. In some cases, though, pH inside the containers was somewhat lower than in the surrounding rumen fluid. In batch cultures, substrate concentration or end-product accumulation might be greater than in typical in vivo feeding situations. Protozoal counts were depleted from continuous culture fermenters faster than expected based on fluid dilution (Ziemer *et al*., 2000). Protozoa in batch culture probably consume more bacteria than would be expected (or probably even possible) in vivo (Jouany, 1996). Evidence such as this seems to support our contention that theories or models developed from in vitro observations and indirect methods to document extensive autolysis of protozoa probably should not be directly extrapolated to in vivo situations.

Protozoa have long been thought to pass from the rumen at a rate slower than ruminal fluid dilution rate (Weller and Pilgrim, 1974). Generation times of protozoa were estimated to be between 9 to 13 hours (Williams and Withers, 1993; Dehority, 1998). This estimate is actually the net doubling time, which is corrected for cell lysis, so true doubling time would have to be shorter to maintain this turnover time (Williams and Withers, 1993). If protozoa and rumen fluid were to pass at the same rate, protozoa with a net generation time of 12 hours would only be maintained if the ruminal fluid dilution rate was less than 0.083/hour (reciprocal of 12 hours), which is slower than measured in many studies, particularly from dairy cattle, which often exceed 0.15/hour (Harvatine *et al*., 2002). In contrast to forage (Figure 1a), when fed mixed forage and grain diets (Figure 1b), counts of entodiniomorphids increased after feeding similarly to isotrichids. After the animal is fed, many protozoa, especially isotrichids, migrate posterior from the reticulum and ventral rumen toward ingested feed and then settle ventrally until the next meal; isotrichids even returned to dorsal sampling sites when the animal was purposely not fed at its normal time (Dehority, 2003). However, the time after feeding (3 hours) seems too short to allow for completion of a cell division to explain the entire increase in counts of entodiniomorphids, also, so chemotaxis to the dorsal sac seems to support our previous assertion of their potential delayed passage from the rumen.

Based on in vitro experiments (Jouany, 1996) and selective faunation experiments (Ivan *et al*., 2000a, 2000b), the entodiniomorphids appear to be more predatory on bacteria than are the isotrichids. Moreover, these authors showed that previously introduced isotrichids decreased considerably in numbers after introduction of other species. Defaunation and selective faunation is very difficult experimentally and renders potential confounding of period effects with treatment effects. Defaunation might be more difficult with cattle than sheep (Towne and Nagaraja, 1990). *Entodinium* species are the most resistant to high grain diets (Towne *et al*., 1990; Hristov *et al*., 2001) and possibly also to high oil (Ivan *et al*., 2001). In a 2 x 2 factorial arrangement of

treatments, only the combination of free oil feeding plus higher grain feeding reduced *Entodinium* generic counts significantly (Ueda *et al*., 2003), again demonstrating their resilience. Thus, the predominance of *Entodinium* in the short term after transfaunation (Williams and Withers, 1993) is not surprising. Dilution from the rumen of protozoa previously labeled with isotope in continuous cultures (Leng and Nolan, 1984) might be subject to the same types of transient adaptations. In contrast, when unsaturated fat was fed to decrease protozoal numbers, isotrichid counts, but not counts of entodiniomorphids, were significantly correlated with efficiency of microbial protein synthesis in cattle (Oldick and Firkins, 2000). Thus, protozoal populations might be affected differently by complete defaunation versus less drastic dietary manipulations to reduce protozoa.

Responses by animals to complete defaunation are mixed. Typically, fiber digestibility is decreased by defaunation, but bacterial N flow and feed efficiency are increased by defaunation (Jouany, 1996; Eugène *et al*., 2004). However, compensatory post-ruminal fiber digestibility might reduce such responses (Ueda *et al*., 2003). Defaunation seems to be more beneficial for average daily gain of animals fed high-forage diets (Eugène *et al*., 2004). Because protozoa excrete large amounts of partially digested bacterial protein (Jouany, 1996), the benefits on animal performance might of course depend on the amount and type of protein fed in the diet. Moreover, a depression in fiber degradability might not quantitatively translate into reduced energy availability if methanogenesis is reduced and VFA are altered (Eugène *et al*., 2004). With all of these qualifications considered, the optimal response might be through decreasing protozoa counts below a threshold to receive some benefits from reduced bacterial predation while maintaining a high enough population for efficient fiber degradation (Williams and Withers, 1993; Jouany, 1996) or for a more stable population. There are a number of potential anti-protozoal agents, ranging from compounds from exotic plants to various fat sources (Hristov *et al*., 2003), although it should be noted that factors such as feeding frequency (Oldick and Firkins, 2000) might influence in vivo responses.

Future studies of protozoal ecology with respect to animal nutrition should be focused on objective reliable identification of most, if not all, predominant protozoal species and on quantification of protozoal biomass in the rumen. In this regard, Karnati *et al.* (2003) first used PCR methods to examine protozoal diversity in rumen samples. They used protozoa-specific primers to achieve amplification of only protozoal 18S rDNA from community DNA. The protozoal rDNA was subsequently cloned, sequenced, and compared to database sequences for identification. In addition to predominant *Entodinium*-like sequences, we found considerable phylogenetic diversity from a limited number of clones sequenced. In contrast, Shin *et al.* (2004) used the same approach but reported that the 18S rDNA sequences were primarily identified as *Entodinium*. Given that some animals fed high grain diets can often have 95 to 99% *Entodinium* species (microscopic counts were not reported) and only 37 sequences were evaluated, lack of diversity of clone sequences might have reflected the true situation. To assess true diversity, more sequences of other *Entodinium* species need to be deposited in databases. Collectively, these two studies document that molecular approaches can be adapted to characterise protozoal populations in the rumen and ultimately should facilitate more reliable and objective analyses of ruminal protozoa independent from morphology characteristics, which are laborious and require complicated differentiation procedures. Alternatively, restriction fragment length polymorphism analysis of the PCR-amplified 18S rRNA gene could identify different ruminal protozoa species, including

those within the same genera or among closely related genera (Regensbogenova *et al*., 2004a). Moreover, because of the numerical predominance of *Entodinium*, species- as well as genus-specific primers are required to effectively uncover the protozoal diversity in the rumen. Given the high sequence identity among different protozoal species, other phylogenetic markers of higher sequence divergence than 18S rDNA, such as 28S rDNA or internal transcribed sequence, should be considered for molecular ecological studies of ruminal protozoa.

As has been used extensively in profiling of bacterial communities, PCR-DGGE should be equally applicable to profiling of protozoal communities. Sylvester *et al.* (2005) and Regensbogenova *et al.* (2004c) demonstrated the utility of PCR-DGGE in profiling protozoal communities in the rumen and duodenum. They designed protozoa-specific primers suitable for PCR-DGGE analysis. They identified the major protozoal species recovered by PCR-DGGE by excision of the intense DGGE bands, re-amplification, and DNA sequencing. This approach will find more application in future ecological studies of rumen protozoa. Moreover, if the 28S rDNA is targeted, more protozoal species should be resolved in PCR-DGGE analysis. Facilitated by these molecular biology techniques, the distribution of different protozoal species or groups in different phases or locations in the rumen of ruminants fed different diets can be examined more accurately.

With respect to the host's nutrition, it is important to quantify protozoal biomass, particularly protein or N, in the rumen and its passage to the small intestine. Microscopic counting can enumerate protozoal populations in the rumen (Dehority, 2003), but it does not allow for measurement of protozoa passage to the duodenum because protozoal cells are lysed in the abomasum (Sylvester *et al*., 2005), and omasal flow has not been determined much in nutrition studies. Protozoal rRNA has been quantified by probe hybridization in ruminal samples in earlier reports based on the difference of eukarya minus fungal 18S rRNA signals (Faichney *et al*., 1997; Krause *et al*., 2000). As discussed previously, quantification of rRNA should be considered semi-quantitative. In addition, in order to convert data to a N basis for nutritional purposes, and particularly to differentiate protozoal N from bacterial N using rRNA or total RNA as a marker, more information is needed on diurnal changes in concentrations of these cell components. In one study (Firkins *et al*., 1987a), both PAB and FAB had similar RNA:N ratios, which were much greater than protozoa, but in another study (Volden *et al*., 1999), protozoal RNA:N ratio (calculated by our summing of reported purine base concentrations) was between that for PAB and FAB. Also, Sylvester *et al.* (2005) described how contaminating bacteria can greatly influence the protozoal N and RNA concentrations.

As molecular-based approaches to assess protozoal populations become adapted more for quantitative purposes, it is essential to determine the average rDNA copies per protozoal cell. In contrast with bacteria (which generally have a constant number of copies of rDNA per cell, although the number varies across species; see earlier discussion), rumen protozoa probably change their rDNA copies per cell over their feeding or life cycle as do the related, non-rumen protozoa (Prescott, 1994; Blomberg *et al*., 1997). Because of the large variance in size among, but even within, protozoal species (Dehority, 1994), the corresponding protozoal biomass probably cannot be accurately determined from counts of protozoa cells. Nevertheless, the average rDNA copies per protozoal cell may be determined from harvested mixtures representative of rumen protozoal communities, and the rDNA copies can be converted to a N basis (Sylvester *et al*.,

2005). In this regard, we reported a procedure to prepare ruminal protozoa samples much more free of bacteria than a standard sedimentation technique (Figure 2). This method also appears to be more representative than a standard sedimentation technique, which can bias the standard mixture towards isotrichids.

Protozoal interactions with *Bacteria* and *Archaea*

It has been known for some time that protozoa predate upon and compete for substrate with the *Bacteria* as well as having synergistic relationships with the methanogenic *Archaea* (Williams and Coleman, 1992), but the complexity of interactions among these microbes has not been well characterised in vivo. Based on in vitro experiments, some protozoa exhibit selective predation on different species of bacteria (Dehority, 2003), including preference toward cellulolytics (Koenig *et al*., 2000). Defaunation of sheep, though, did not significantly change numbers of culturable cellulolytic bacteria, whereas refaunation decreased the cellulolytics below those observed in the original faunation period (Koenig *et al*., 2000). The FAB have been proposed to be physically more susceptible to predation than PAB (McAllister *et al*., 1994), which tend to contain more adherent cellulolytics (see earlier discussion), so interactions between protozoa and bacteria might be more complex in vivo than in vitro and might take longer to fully establish than allowed in most experimental designs. Protozoa have been estimated to contribute 1/4 to 1/3 of the total fiber digestibility in the rumen (Williams and Coleman, 1997). Thus, the trends for defaunation to consistently decrease fiber digestibility (Eugène *et al*., 2004) imply either that protozoa contribute more to fibrolysis than do the bacteria that replace them after defaunation or that protozoa provide a synergistic action with fibrolytic bacteria in the rumen that provides greater benefit than the negative effect of predation on cellulolytic bacteria. Michalet-Doreau *et al.* (2001) reported that the higher specific fibrolytic activity associated with firmly adherent PAB was a result of washing to remove protozoa (presumed to have a lower fibrolytic activity than

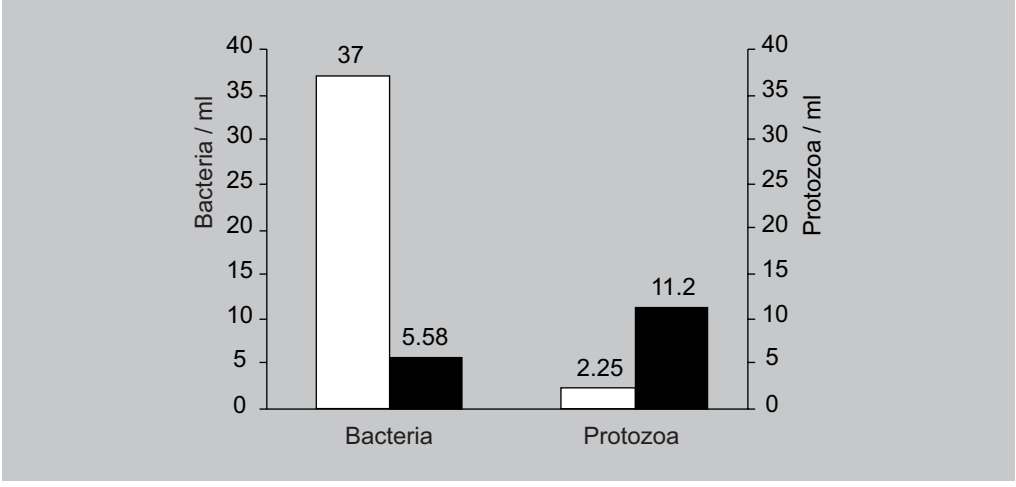

Figure 2. Bacterial (x 10^9) and protozoal (x10^6) cell counts in protozoal samples harvested by sedimentation (open bars) or filtration (filled bars).

PAB); thus more efforts should explore the potentially positive aspects of protozoa on fibrolytic bacteria. Harboring prokaryotic populations both extra- and intra-cellularly, protozoa have well established syntrophic interactions with bacteria and especially methanogens (Hegarty, 1999). Although no comparable direct synergism stands out with fibrolytic bacteria, the synergism could also be indirect. Faunated animals consistently have higher ruminal ammonia concentrations (Eugène *et al*., 2004), which might stimulate the ammonia-requiring cellulolytics (Dehority, 2003). Also, protozoa might scavenge O_2, thus benefiting strictly anaerobic prokaryotes in the rumen (Hegarty, 1999; Dehority, 2003).

The rumen methanogens are very important to animal nutrition because they influence the populations of bacterial and protozoal hydrogen producers (Wolin *et al*., 1997). By shifting fermentation toward acetate and from propionate, methanogenesis decreases the conversion of digestible energy to metabolisable energy and also contributes to the emission of methane, a deleterious greenhouse gas. Although these topics are important, our discussion will be limited to methanogen populations and their potential interactions with protozoa and bacteria.

With fastidious growth conditions, some methanogens are difficult to grow in laboratories but can be detected readily with molecular biology methods (Tokura *et al*., 1999). Methanogen numbers can be estimated by quantifying archaeal rRNA using hybridization (Sharp *et al*., 1998) or determined more directly by counting cells labeled with fluorescent probes (fluorescent in situ hybridization, FISH) specific to total methanogens or particular orders (Machmüller *et al*., 2003). Methanogens appear to vary in abundance considerably during a feeding cycle and even have varying association with protozoa (Baker, 1999), perhaps related to the levels of H_2 production by the latter. Interestingly, total methanogen numbers were not necessarily correlated with total rRNA copies or the amount of methane production (Machmüller *et al*., 2003). Methanogenesis in the rumen is probably controlled by H_2 availability rather than methanogen abundance (Hegarty, 1999). Baker (1999) also discussed the potential uncoupling of methanogenesis with anabolic reactions. Methanogenesis generally is reduced by about 15% by defaunation (Hegarty, 1999; Machmüller *et al*., 2003). However, defaunation might be more beneficial with higher grain diets, which might increase the production of H_2 by protozoa (Hegarty, 1999). Protozoa also provide some advantage by quenching oxygen through their oxygen-tolerant hydrogenosomes, or they simply provide a vehicle for retention of slower-growing methanogens in the rumen (Williams and Coleman, 1997).

Several studies have quantified various orders of methanogens under different conditions. Rusitec fermentation vessels have a semi-continuous outflow of particles that allows retention of protozoa (Dohme *et al*., 2001). This study showed that medium chain fatty acids and linoleic acid significantly decreased protozoal counts. However, effects of fatty acids on methanogen counts or methanogenesis were not conclusive because of variation in measurements. More recently, Soliva *et al*. (2004a) reported that a combinations of lauric and myristic acids inhibited *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* families, but not *Methanococcales*, in Rusitec. In batch cultures, increasing concentrations of lauric acid decreased methanogenesis and the numbers of all four methanogen orders by similar degrees (Soliva *et al*., 2003). The authors suggested that myristic acid potentiated the response. Soliva *et al*. (2004a, 2004b) discussed reasons for variation in the concentrations of methanogen orders among different

studies, including animal and inoculum effects, length of incubation, different quantification procedures, and whether or not the batch cultures were gassed with H_2. In another study with sheep but using similar methods (Machmüller *et al*., 2003), the same group was not able to make differential counts of these orders because freezing and thawing disrupted methanogen cells. However, they estimated the abundance of these orders by quantifying their 16S rRNA copies. They found that defaunation actually increased methanogen abundance, especially when coconut oil (high in lauric acid) was fed. In contrast, defaunation tended to increase copies of 16S rRNA from *Methanococcales* and *Methanobacteriales* when inert prilled fat was fed, but it dramatically decreased the rRNA copies from these methanogen orders when coconut oil was fed. The rRNA copies from *Methanomicrobiales* and *Methanosarcinales* were not affected by defaunation or fat source.

In continuous culture vessels that rapidly wash out protozoa, the percentage of 16S rRNA of *Archaea* origin remained the same (Sharp *et al*., 1998) or increased (Ziemer *et al*., 2000) after inoculation. However, Sharp *et al.* (1998) showed dramatic shifts in the methanogen orders coinciding with losses of protozoa from fermenters (quantified using total *Eukarya* probes). The rRNA hybridization signal targeting the family *Methanobacteriaceae* (in the *Methanobacteriales* order) was predominant in the original rumen fluid (92% of the *Archaea* signal) and particularly in a protozoal fraction (99%), but it decreased to 73% in the fermenters after protozoa were washed out. *Methanomicrobiales* accounted for most of the remainder of the total archaeal signal. Thus, the authors concluded that *Methanobacteriales* can be free-living or associated with protozoa, but the *Methanomicrobiales* appear to be predominantly free-living, increasing in numbers to replace the protozoal-associated methanogens. In sheep that were monofaunated with *Isotricha prostoma*, *Eudiplodinium maggii*, or *Polyplastron multivesiculatum*, the associated methanogens were similar among animals and primarily grouped with *Methanobrevibacter* species of *Methanobacteriaceae* (Chagan *et al*., 1999). In a recent study (Regensbogenova *et al*., 2004b) in which DNA was extracted from single cells of rumen protozoal species, however, there appeared to be no consistent pattern in methanogen sequences recovered from protozoa, suggesting non-specific association between protozoa and methanogens. Moreover, novel sequences were produced, and the sequences did not cluster with those from previous studies. Further work is needed to fully describe protozoa-archaea interactions. As these procedures advance to DNA-based quantification, for reasons described previously, caution must be used when adapting RNA-based probes for primers for use in PCR (Skillman *et al*., 2004).

The phylogenetic diversity of *Archaea* has been demonstrated recently in clone libraries constructed from sheep grazing pasture or consuming dry forages and concentrates (Wright *et al*., 2004). Diversity was greatest for sheep grazing pasture, with 33 different phylotypes of methanogens identified. Of a total 733 clones sequenced from clone libraries prepared, 30 phylotypes appeared most closely related to *Methanobrevibacter* (in the family *Methanobacteriaceae*), seeming to support the high prevalence of this genus determined using rRNA probes (Sharp *et al*., 1998). No sequence was found related to *Methanococcales*. Many of these clones formed distinct clusters associated with isolates of either *M. smithii* or *M. ruminantium*. They also noted several other distinct sequences, including two that could be from new species and possibly even a new order, suggesting undescribed methanogens in the rumen. Tajima *et al.* (2001b) also provided evidence for existence of a new species of hydrogen-scavenging methanogen. Although inoculation with

soil methanogens possibly could be more prevalent for grazing ruminants, these studies seem to collectively indicate that the majority of the diversity is due to endemic methanogens adapted to the rumen environment, perhaps to different niches therein. Although the latter authors commented on the apparent lower diversity of *Archaea* with respect to *Bacteria*, there are some differences in methanogen populations among animals (Wright *et al*., 2004), and perhaps greater diversity will emerge in future studies as more information from varying geographical regions becomes available.

These studies collectively support the importance of *Methanobacteriales* and its apparent relationship with protozoa, but the role of *Methanococcales*, though numerically predominant as determined in several studies (Soliva *et al*., 2004b), is not clear. Perhaps some of the discrepancy can be related to irregularities in 16S rDNA sequences among methanogens (Wright and Pimm, 2003). Sampling times with respect to feeding, locations in the rumen, and other factors related to ruminal ecology of *Archaea* are largely unexplored. Given their close association with protozoa (Sharp *et al*., 1998) and the apparent discrepancies between methanogen counts and methanogenesis (Machmüller *et al*., 2003), more studies are needed to relate numbers of specific methanogens to the amount of methane produced. Future researchers should consider the quantitative issues discussed previously for *Bacteria*. In addition, they should consider the potential adaptations developing over time after introduction of anti-protozoa or anti-methanogen agents. Increased knowledge of diversity of methanogens and protozoa as well as their interactions in the rumen should guide our efforts to minimize methane emission from ruminants and improve efficiency of microbial protein synthesis.

Conclusions

Molecular biology techniques have greatly advanced our perspective of rumen microbial ecology; however, due to the limited number of sequences retrieved and characterised with respect to known isolates, our understanding of the diversity and interactions among protozoa, *Bacteria*, and *Archaea* is still limited. Alteration of these communities caused by dietary manipulations appears relatively smaller than might be expected; and differences among animals fed similar diets, relatively larger. Such small changes in microbial diversity and community structure are likely below the detection limits of conventional molecular techniques, such as DGGE and clone libraries. Future studies should be directed toward comprehensive analysis of rumen microbial communities using high throughput technologies, such as SARST-v1. Besides increasing the number of sequences needed to statistically verify microbial diversity, such procedures could help focus more targeted efforts to quantify functional groups. Quantitative assays of high sensitivity, such as real-time PCR, should be developed to quantify abundance and examine dynamics of individual populations, using species-, genus-, or guild-specific primers.

With more powerful and systematically available molecular techniques, there is increasing availability to integrate microbiological and nutritional objectives in future research. Increasing characterisation of diversity and function of microbial communities should allow more opportunities to account for variation among diets, animals, and experiments for better prediction of rumen digestibility of nutrients and efficiency of microbial growth. These coordinated efforts

should help us meet these goals while accounting for increasing societal demands for improved efficiency of nutrient usage and more desirable animal products.

Acknowledgements

For excellent discussions and collaborations, we thank Burk Dehority, Sanjay Karnati, Mark Morrison, Jamie Newbold, Carine Reveneau, and John Sylvester. Research support by the Ohio Agricultural and Research Development Center and by the USDA National Research Initiative Competitive Grants Program is gratefully acknowledged.

References

Akin, D.E. & I.F.E. Barton (1983). Rumen microbial attachment and degradation of plant cell walls. Federation Proceedings, 42, 114-121.

Allen, M.S. (1997). Relationship between fermentation acid production in the rumen and the requirement for physically effective fiber. Journal of Dairy Science, 80, 1447-1462.

Ankrah, P., S.C. Loerch & B.A. Dehority (1990). Sequestration, migration and lysis of protozoa in the rumen. Journal of General Microbiology, 136, 1869-1875.

Baker, S.K. (1999). Rumen methanogens, and inhibition of methanogenesis. Australian Journal of Agricultural Research, 50, 1293-1298.

Balcells, J., F. Zakraoui & C. Castrillo (1998). Quantification and chemical composition of mixed bacteria harvested from solid fractions of rumen digesta: Effect of detachment procedure. Animal Feed Science and Technology, 71, 269-282.

Blomberg, P., C. Randolph, C.-H. Yao & M.-C. Yao (1997). Regulatory sequences for the amplification and replication of the ribosomal DNA minichromosome in *Tetrahymena thermophila*. Molecular and Cellular Biology, 17, 7237-7247.

Boschker, H.T.S., S.C. Nold, P. Wellsbury, D. Bos, W. de Graaf, W. Pel, J. Parkes & T.E. Cappenberg (1998). Direct linking of microbial populations to specific biogeochemical proceses by 13C-labeling of biomarkers. Nature, 392, 801-805.

Bowman, J.G. P. & J.L. Firkins (1993). Effects of forage species and particle size on bacterial cellulolytic activity and colonization in situ. Journal of Animal Science, 71, 1623-1633.

Briesacher, S.L., T. May, K.N. Grigsby, M.S. Kerley, R.V. Anthony & J.A. Paterson (1992). Use of DNA probes to monitor nutritional effects of ruminal prokaryotes and *Fibrobacter succinogenes* S85. Journal of Animal Science, 70, 289-295.

Cafe, L.M. & D.P. Poppi (1994). The fate and behavior of imbibed water in the rumen of cattle. Journal of Agricultural Science (Cambridge), 122, 139-144.

Calsamiglia, S., A. Ferret & M. Devant (2002). Effects of pH and pH fluctuations on microbial fermentation and nutrient flow from a dual-flow continuous culture system. Journal of Dairy Science, 85, 574-579.

Candela, M., B. Vitali, D. Matteuzzi & P. Brigidi (2004). Evaluation of the *rrn* operon copy number in *Bifidobacterium* using real-time PCR. Letters in Applied Microbiology, 38, 229-232.

Chagan, I., M. Tokura, J.P. Jouany & K. Ushida (1999). Detection of methanogenic archaea associated with rumen ciliate protozoa. Journal of General and Applied Microbiology, 45, 305-308.

Chan, W.W. & B.A. Dehority (1999). Production of *Ruminococcus flavefaciens* growth inhibitor(s) by *Ruminococcus albus*. Animal Feed Science and Technology, 77, 61-71.

Chen, J., D.M. Stevenson & P.J. Weimer (2004). Albusin B, a bacteriocin from the ruminal bacterium *Ruminococcus albus* 7 that inhibits growth of *Ruminococcus flavefaciens*. Applied and Environmental Microbiology, 70, 3167-3170.

Cheng, K.-J. & T.A. McAllister (1997). Compartmentalization in the rumen. In: Hobson, P. N. & C. S. Stewart (eds) The Rumen Microbial Ecosystem. Blackie Academic & Professional, New York, NY, pp. 492-522.

Cookson, A.L., S.J. Noel, W.J. Kelly & G.T. Attwood (2004). The use of PCR for the identification and characterisation of bacteriocin genes from bacterial strains isolated from rumen or caecal contents of cattle and sheep. FEMS Microbiology and Ecology, 48, 199-207.

Cotta, M.A. & J.B. Russell (1997). Digestion of nitrogen in the rumen: A model for metabolism of nitrogen compounds in gastrointestinal environments. In: Mackie, R.I. & B.A. White (eds) Gastrointestinal Ecosystems and Fermentations, vol. 1. Chapman and Hall, New York, NY, pp. 380-423.

Crosby, L.D. & C.S. Criddle (2003). Understanding bias in microbial community analysis techniques due to *rrn* operon copy number heterogeneity. BioTechniques, 34, 790-802.

Dehority, B.A. (2003). Rumen Microbiology. Nottingham University Press, Nottingham, UK.

Dehority, B.A. (1994). Rumen ciliate protozoa of the blue duiker (*Cephalophus monticola*), with observations on morphological variation lines within the species *Entodinium dubardi*. Journal of Eukaryotic Microbiology, 41, 103-111.

Dehority, B. A. (1998). Generation times of *Epidinium caudatum* and *Entodinium caudatum*, determined in vitro by transferring at various time intervals. Journal of Animal Science, 76, 1189-1196.

Dehority, B.A., P.A. Tirabasso & A.P. Grifo, Jr. (1989). Most-probable-number procedures for enumerating ruminal bacteria, including the simultaneous estimation of total and cellulolytic numbers in one medium. Applied and Environmental Microbiology, 55, 2789-2792.

Dijkstra, J., J. France & D.R. Davies (1998). Different mathematical approaches to estimating microbial protein supply in ruminants. Journal of Dairy Science, 81, 3370-3384.

Dohme, F., A. Machmüller, A. Wasserfallen & M. Kreuzer (2001). Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminant diets. Letters in Applied Microbiology, 32, 47-51.

Eugène, M., H. Archimède & D. Sauvant (2004). Quantitative meta-analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. Livestock Production Science, 85, 81-97.

Faichney, G.J., C. Poncet, B. Lassalas, J.P. Jouany, L. Millet, J. Dore & A.G. Brownlee (1997). Effects of concentrates in a hay diet on the contribution of anaerobic fungi, protozoa and bacteria to nitrogen in rumen and duodenal digesta in sheep. Animal Feed Science and Technology, 64, 193-213.

Fields, M.W., S. Mallik & J.B. Russell (2000). *Fibrobacter succinogenes* S85 ferments ball-milled cellulose as fast as cellobiose until cellulose surface area is limiting. Applied Microbiology and Biotechnology, 54, 570-574.

Firkins, J.L. (1996). Maximizing microbial protein synthesis in the rumen. Journal of Nutrition, 126, 1347S-1354S.

Firkins, J.L., M.S. Allen, B.S. Oldick & N.R. St-Pierre (1998). Modeling ruminal digestibility of carbohydrates and microbial protein flow to the duodenum. Journal of Dairy Science, 81, 3350-3369.

Firkins, J.L., L.L. Berger, N.R. Merchen, G.C. Fahey & R.L. Mulvaney (1987a). Ruminal nitrogen metabolism in steers as affected by feed intake and dietary urea concentration. Journal of Dairy Science, 70, 2302-2311.

Firkins, J.L., S.M. Lewis, L. Montgomery, L.L. Berger, N.R. Merchen & G.C. Fahey (1987b). Effects of feed intake and dietary urea concentration on ruminal dilution rate and efficiency of bacterial growth in steers. Journal of Dairy Science, 70, 2312-2321.

Firkins, J.L. & C.K. Reynolds (2005). Whole animal nitrogen balance in cattle. In: Hristov, A.N. & E. Pfeffer (eds) Nitrogen and Phosphorus Nutrition of Cattle and Environment. CAB International, Wallingford, U.K., pp. 167-185.

Fogel, G.B., C.R. Collins, J.Li & C.F. Brunk (1999). Prokaryotic genome size and SSU rDNA copy number: Estimation of microbial relative abundance from a mixed population. Microbial Ecology, 38, 93-113.

Fondevila, M. & B.A. Dehority (2001). In vitro growth and starch digestion by *Entodinium exiguum* as influenced by the presence or absence of live bacteria. Journal of Animal Science, 79, 2465-2471.

Goldman, N. (1996). Phylogenetic estimation. In: Bishop, M. J. & C. J. Rawling (eds) DNA and Protein Sequence Analysis. IRL Press, Oxford, U.K., pp. 279-312.

Hahn, M.W., P. Stadler, Q.L. Wu & M. Pöckl (2004). The filtration-acclimatization method for isolation of an important fraction of the not readily cultivable bacteria. Journal of Microbiological Methods, 57, 379-390.

Harfoot, C.G. & G.P. Hazlewood (1997). Lipid metabolism in the rumen. In: Hobson, P.N. & C.S. Stewart (eds) The Rumen Microbial Ecosystem. Blackie Academic & Professional, New York, NY, pp. 382-426.

Harvatine, D.I., J.E. Winkler, M. Devant-Guille, J.L. Firkins, N.R. St-Pierre & M.L. Eastridge (2002). Whole linted cottonseed as a forage substitute: Fiber effectiveness and digestion kinetics. Journal of Dairy Science, 85, 1988-1999.

Hegarty, R.S. (1999). Reducing rumen methane emissions through elimination of rumen protozoa. Australian Journal of Agricultural Research, 50, 1321-1327.

Hespell, R.B., D.E. Akin & B.A. Dehority (1997). Bacteria, fungi, and protozoa of the rumen. In: Mackie, R.I., B.A. White & R.E. Isaacson (eds) Gastrointestinal Microbiology, vol. 2. Chapman and Hall, New York, pp. 59-141.

Hocquette, J.-H. & A.M. Brandstetter (2002). Common practice in molecular biology may introduce statistical bias and misleading biological interpretation. Journal of Nutritional Biochemisty, 13, 370-377.

Holben, W.E., K.P. Feris, A. Kettunen & J.H.A. Apajalahti (2004). GC fractionation enhances microbial community diversity assessment and detection of minority populations of bacteria by denaturing gradient gel electrophoresis. Applied and Environmental Microbiology, 70, 2263-2270.

Hristov, A.N., M. Ivan, L. Neill & T.A. McAllister (2003). Evaluation of several potential bioactive agents for reducing protozoal activity in vitro. Animal Feed Science and Technology, 105, 163-184.

Hristov, A.N., M. Ivan, L.M. Rode & T.A. McAllister (2001). Fermentation characteristics and ruminal ciliate protozoal populations in cattle fed medium- or high-concentrate barley-based diets. Journal of Animal Science, 79, 515-524.

Hughes, J.B., J.J. Hellmann, T.H. Ricketts & B.J. M. Bohannan (2001). Counting the uncountable: Statistical approaches to estimating microbial diversity. Applied and Environmental Microbiology, 67, 4399-4406.

Hunter-Cevera, J.C. (1998). The value of microbial diversity. Current Opinion in Microbiology, 1, 278-285.

Ito, A., Y. Miyazaki & S. mai (2001). Light microscopic observation of infraciliature and morphogenesis in six species of rumen *Ostracodinium* ciliates. Journal of Eukaryotic Microbiology, 48, 440-448.

Ivan, M., P.S. Mir, K.M. Koenig, L.M. Rode, L. Neill, T. Entz & Z. Mir (2001). Effects of dietary sunflower seed oil on rumen protozoa population and tissue concentration of conjugated linoleic acid in sheep. Small Ruminant Research, 41, 215-227.

Ivan, M., L. Neill & T. Entz (2000a). Ruminal fermentation and duodenal flow following progressive inoculations of fauna-free wethers with major individual species of ciliate protozoa or total fauna. Journal of Animal Science, 78, 750-759.

Ivan, M., L. Neill, R. Forster, R. Alimon, L.M. Rode & T. Entz (2000b). Effects of *Isotricha*, *Dasytricha*, *Entodinium*, and total fauna on ruminal fermentation and duodenal flow in whethers fed different diets. Journal of Dairy Science, 83, 776-787.

Jouany, J.P. (1996). Effect of rumen protozoa on nitrogen utilization by ruminants. Journal of Nutrition, 126, 1335S-1346S.

Kaeberlein, T., K. Lewis & S.S. Epstein (2002). Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. Science, 296, 1127-1129.

Kalmokoff, M.L., T.D. Cyr, M.A. Hefford, M.F. Whitford & R.M. Teather (2003). Bacteriocin AR10, a new cyclic bacteriocin produced by the ruminal anaerobe *Butyrivibrio fibrisolvens* AR10: characterization of the gene and peptide. Canadian Journal of Microbiology, 49, 763-773.

Karnati, S. K. R., Z. Yu, J. T. Sylvester, B. A. Dehority, M. Morrison & J. L. Firkins (2003). Technical note: Specific PCR amplification of protozoal 18S rDNA sequences from DNA extracted from ruminal samples of cows. Journal of Animal Science, 81, 812-815.

Klieve, A.V., D. Hennessey, D. Ouwerkerk, R.J. Forster, R.I. Mackie & G.T. Attwood (2003). Establishing populations of *Megasphaera elsdenii* YE34 and *Butyrivibrio fibrisolvens* YE44 in the rumen of cattle fed high grain diets. Journal of Applied Microbiology, 95, 621-630.

Kocherginskaya, S., R.I. Aminov & B.A. White (2001). Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches. Anaerobe, 7, 119-134.

Koenig, K.M., C.J. Newbold, F.M. Mcintosh & L.M. Rode (2000). Effects of protozoa on bacterial nitrogen recycling in the rumen. Journal of Animal Science, 78, 2431-2445.

Koike, S. & Y. Kobayashi (2001). Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. FEMS Microbiolology Letters, 204, 361-366.

Koike, S., Y. Kobayashi & K. Tanaka (2003a). Kinetics of in sacco fiber-attachment of representative cellulolytic bacteria monitored by competitive PCR. Journal of Dairy Science, 86, 1429-1435.

Koike, S., S. Yoshitani, Y. Kobayashi & K. Tanaka (2003b). Phylogenetic analysis of fiber-associated rumen bacterial community and PCR detection of uncultured bacteria. FEMS Microbiolology Letters, 229, 23-30.

Krause, D.O., R.J. Bunch, W.J.M. Smith & C. S. McSweeney (1999a). Diversity of *Ruminococcus* strains: A survey of genetic polymorphisms and plant digestibility. Journal of Applied Microbiology, 86, 487-495.

Krause, D.O., B.P. Dalrymple, W.J. Smith, R.I. Mackie & C.S. McSweeney (1999b). 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep. Microbiology, 145, 1797-1807.

Krause, D.O., S.E. Denman, R.I. Mackie, M. Morrison, A.L. Rae, G.T. Attwood & C.S. McSweeney (2003). Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. FEMS Microbiology Reviews, 27, 663-693.

Krause, D.O. & J.B. Russell (1996). How many ruminal bacteria are there? Journal of Dairy Science, 79, 1467-1475.

Krause, D.O., W.J.M. Smith, F.M.E. Ryan, R.I. Mackie & C.S. McSweeney (2000). Use of 16S-rRNA based techniques to investigate the ecological succession of microbial populations in the immature lamb rumen: Tracking of a specific strain of inoculated *Ruminococcus* and interactions with other microbial populations *in vivo*. Microbial Ecology, 38, 365-376.

Lapierre, H. & G.E. Lobley (2001). Nitrogen recycling in the ruminant: A review. Journal of Dairy Science, 84(E. Suppl.), E223-E236.

Larue, R., Z. Yu, V.A. Parisi, A.R. Egan & M. Morrison (2005). Novel microbial diversity adherent to plant biomass in the herbivore gastrointestinal tract, as revealed by ribosomal intergenic spacer analysis and *rrs* gene sequencing. Environmental Microbiology, (in press).

Leedle, J.A.Z., M.P. Bryant & R.B. Hespell (1982). Diurnal variations in bacterial numbers and fluid parameters in ruminal contents of animals fed low- or high-forage diets. Applied and Environmental Microbiology, 44, 402-412.

Leng, R.A. & J.V. Nolan (1984). Nitrogen metabolism in the rumen. Journal of Dairy Science, 67, 1072-1089.

Machmüller, A., C.R. Soliva & M. Kreuzer (2003). Effect of coconut oil and defaunation treatment on methanogenesis in sheep. Reproduction Nutrition and Development, 43, 41-55.

Mackie, R.I., R.I. Aminov, B.A. White & C.S. McSweeney (2000). Molecular ecology and diversity in gut microbial ecosystems. In: Cronjé, P.B. (ed) Ruminant Physiology. Digestion, Metabolism, Growth, and Reproduction. CABI Publishing, New York, NY, pp. 61-77.

Martin, C., E. Devillard & B. Michalet-Doreau (1999). Influence of sampling site on concentrations and carbohydrate-degrading enzyme activities of protozoa and bacteria in the rumen. Journal of Animal Science, 77, 979-987.

Mathison, G.W., E.K. Okine, A.S. Vaage, M. Kaske & L.P. Milligan (1995). Current understanding of the contribution of the propulsive activities in the forestomach to the flow of digesta. In: von Engelhardt, W., S. Leonhard-Marek, G. Breves & D. Giesecke (eds) Ruminant Physiology. Digestion, Metabolism, Growth and Reproduction. Proc. Eighth Int. Symp. on Ruminant Physiol. Ferdinand Enke Verlag, Stuttgart, Germany, pp. 23-41.

McAllister, T.A., H.D. Bae, G.A. Jones & K.-J. Cheng (1994). Microbial attachment and feed digestion in the rumen. Journal of Animal Science, 72, 3004-3018.

McBride, G.B., J.L. McWhirter & M.H. Dalgety (2003). Uncertainty in most probable number calculations for microbiological assays. Journal of AOAC International, 86, 1084-1088.

Michalet-Doreau, B., I. Fernandez & G. Fonty (2002). A comparison of enzymatic and molecular approaches to characterize the cellulolytic microbial ecosystems of the rumen and the cecum. Journal of Animal Science, 80, 790-796.

Michalet-Doreau, B., I. Fernandez, C. Peyron, L. Millet & G. Fonty (2001). Fibrolytic activities and cellulolytic bacterial community structure in the solid and liquid phases of rumen contents. Reproduction Nutrition and Development, 41, 187-194.

Michalowski, T. (1998). A proposed simple method for determining the outflow of ciliates from the reticulo-rumen. Journal of Animal and Feed Science, 7, 83-92.

Michalowski, T., J. Harmeyer & G. Breves (1986). The passage of protozoa from the reticulo-rumen through the omasum of sheep. British Journal of Nutrition, 56, 625-634.

Minato, H., S. Ishibashi & T. Hamaoka (1988). Cellular fatty acid and sugar composition of representative strains of rumen bacteria. Journal of General and Applied Microbiology, 34, 303-319.

Miron, J., D. Ben-Ghedalia & M. Morrison (2001). Invited review: Adhesion mechanisms of rumen cellulolytic bacteria. Journal of Dairy Science, 84, 1294-1309.

Moon, M. & K.L. Anderson (2001). *Eubacterium cellulosolvens* alters its membrane protein, lipoprotein, and fatty acid composition in response to growth on cellulose. Anaerobe, *7*, 227-236.

Moré, M.I., J.B. Herrick, M.C. Silva, W.C. Ghiorse & E.L. Madsen (1994). Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. Applied and Environmental Microbiology, 60, 1572-1580.

Mouriño, F., R. Akkarawongsa & P.J. Weimer (2001). Initial pH as a determinant of cellulose digestion rate by mixed ruminal microorganisms in vitro. Journal of Dairy Science, 84, 848-859.

Neufeld, J.D., Z. Yu, W. Lam & W.W. Mohn (2004). Serial analysis of ribosomal sequence tags (SARST): a high-throughput method for profiling complex microbial communities. Environmental Microbiology, 6, 131-144.

Nsabimana, E., S. Kisidayova, D. Macheboeuf, C.J. Newbold & J.P. Jouany (2003). Two-step procedure for cryopreservation of rumen ciliates, an effective tool for creation of a frozen rumen protozoa bank. Applied and Environmental Microbiology, 69, 3862-3832.

Oldick, B.S. & J.L. Firkins (2000). Effects of degree of fat saturation on fiber digestion and microbial protein synthesis when diets are fed twelve times daily. Journal of Animal Science, 78, 2412-2420.

Oldick, B.S., J.L. Firkins & N.R. St-Pierre (1999). Estimation of microbial nitrogen flow to the duodenum of cattle based on dry matter intake and diet composition. Journal of Dairy Science, 82, 1497-1511.

Ouwerkerk, D., A.V. Klieve & R.J. Forster (2002a). Corrigendum. Journal of Applied Microbiology, 93, 913.

Ouwerkerk, D., A.V. Klieve & R.J. Forster (2002b). Enumeration of *Megasphaera elsdenii* in rumen contents by real-time Taq nuclease assay. Journal of Applied Microbiology, 92, 753-758.

Peterson, D.G., E.A. Matitashvili & D. E. Bauman (2003). Diet-induced milk fat depression in dairy cows results in increased *trans*-10, *cis*-12 CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis. Journal of Nutrition, 133, 3098-3102.

Polz, M.F., S. Bertilsson, S.G. Acinas & D. Hunt (2003). A(r)ray of hope in analysis of the function and diversity of microbial communities. Biol. Bull., 204, 196-199.

Polz, M.F. & C.M. Cavanaugh (1998). Bias in template-to-product ratios of multitemplate PCR. Applied and Environmental Microbiology, 64, 3724-3730.

Prescott, D.M. (1994). The DNA of ciliated protozoa. Microbiological Reviews, 58, 233-267.

Punia, B.S. & J. Leibholz (1994). Effect of level of intake of kikuyu (*Pennisetum clandestinum*) grass hay on the flow of protozoal nitrogen to the omasum of cattle. Animal Feed Science and Technology, 47, 77-87.

Raskin, L., W.C. Capman, R. Sharp, L.K. Poulsen & D.A. Stahl (1997). Molecular ecology of gastrointestinal ecosystems. In: Mackie, R. I., B. A. White & R. E. Isaacson (eds) Gastrointestinal Microbiology, Vol. 2. Gastrointestinal Microbes and Host Interactions. Chapman Hall, New York, pp. 243-298.

Regensbogenova, M., S. Kisidayova, T. Michalowski, P. Javorsky, S.Y. Moon-van der Staay, G.W.M. Moon-van der Stay, J.H.P. Hackstein, N.R. McEwan, J.P. Jouany, C.J. Newbold & P. Pristas (2004a). Rapid identification of rumen protozoa by restriction analysis of amplified 18S rRNA gene. Acta Protozoologica, 43, 219-224.

Regensbogenova, M., N.R. McEwan, P. Javorsky, S. Kisidayova, T. Michalowski, C.J. Newbold, J.H.P. Hackstein & P. Pristas (2004b). A re-appraisal of the diversity of the methanogens associated with the rumen ciliates. FEMS Microbiolology Letters, 238, 307-313.

Regensbogenova, M., P. Pristas, P. Javorsky, S.Y. Moon-van der Staay, G.W.M. van der Staay, J.H.P. Hackstein, C.J. Newbold & N.R. McEwan (2004c). Assessment of ciliates in the sheep rumen by DGGE. Letters in Applied Microbiology, 39, 144-147.

Reilly, K., V.R. Carruthers & G.T. Attwood (2002). Design and use of 16S ribosomal DNA-directed primers in competitive PCRs to enumerate proteolytic bacteria in the rumen. Microbial Ecology, 43, 259-270.

Rosselló-Mora, R. & R. Amann (2001). The species concept for prokaryotes. FEMS Microbiology Reviews, 25, 39-67.

Rychlik, J.L. & J.B. Russell (2002). Bacteriocin-like activity of *Butyrivibrio fibrisolvens* JL5 and its effect on other ruminal bacteria and ammonia productin. Applied and Environmental Microbiology, 68, 1040-1046.

Schmittgen, T.D., B.A. Zakrajsek, A.G. Mills, V. Gorn, M.J. Singer & M.W. Reed (2000). Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: Comparison of endpoint and real-time methods. Analytical Biochemistry, 285, 194-204.

Sharp, R., C.J. Ziemer, M.D. Stern & D.A. Stahl (1998). Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microbiology and Ecology, 26, 71-78.

Shi, Y., C.L. Odt & P.J. Weimer (1997). Competition for cellulose among three predominant ruminal cellulolytic bacteria under substrate-excess and substrate- limited conditions. Applied and Environmental Microbiology, 63, 734-742.

Shin, E.C., K.M. Cho, W.J. Lim, S.Y. Hong, C.L. An, E.J. Kim, Y.K. Kim, B.R. Choi, J.M. An, J.M. Kang, H. Kim & H.D. Yun (2004). Phylogenetic analysis of protozoa in the rumen contents of cow based on the 18S rDNA sequences. Journal of Applied Microbiology, 97, 378-383.

Skillman, L.C., P.N. Evans, G.E. Naylor, B. Morvan, G.N. Jarvis & K.N. Joblin (2004). 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. Anaerobe, 10, 277-285.

Soliva, C.R., I.K. Hindrichsen, L. Meile, M. Kreuzer & A. Machmüller (2003). Effects of mixtures of lauric and myristic acid on rumen methanogens and methanogenesis *in vitro*. Letters in Applied Microbiology, 37, 35-39.

Soliva, C.R., L. Meile, I.K. Hindrichsen, M. Kreuzer & A. Machmüller (2004a). Myristic acid supports the immediate inhibitory effect of lauric acid on ruminal methanogens and methane release. Anaerobe, 10, 269-276.

Soliva, C.R., L. Meile, M. Kreuzer & A. Machmüller (2004b). Rumen simulation technique study on the interactions of dietary lauric and myristic acid supplementation in suppressing ruminal methanogenesis. British Journal of Nutrition, 92, 689-700.

Stern, M.D., G.A. Varga, J.H. Clark, J.L. Firkins, J.T. Huber & D.L. Palmquist (1994). Evaluation of chemical and physical properties of feeds that affect protein metabolism in the rumen. Journal of Dairy Science, 77, 2762-2786.

Stevenson, B. S., S. A. Eichorst, J. T. Wertz, T. M. Schmidt & J. A. Breznak (2004). New strategies for cultivation and detection of previously uncultured microbes. Applied and Environmental Microbiology, 70, 4748-4755.

Stewart, C.S., H.J. Flint & M.P. Bryant (1997). The rumen bacteria. In: Hobson, P.N. & C.S. Stewart (eds) The Rumen Microbial Ecosystem. Blackie Academic & Professional, New York, pp. 10-72.

Sylvester, J.T., S.K.R. Karnati, Z. Yu, M. Morrison & J.L. Firkins (2004). Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. Journal of Nutrition, 134, 3378-3384.

Sylvester, J.T., S.K.R. Karnati, Z. Yu, C.J. Newbold & J.L. Firkins (2005). Evaluation of a real-time PCR assay for measuring the ruminal pool and duodenal flow of protozoal nitrogen. Journal of Dairy Science, 88, 2083-2095.

Tajima, K., R.I. Aminov, H. Nagamine, H. Matsui, M. Nakamura & Y. Benno (2001a). Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Applied and Environmental Microbiology, 67, 2766-2774.

Tajima, K., R.I. Aminov, T. Nagamine, K. Ogata, K. Nakamura, H. Matsui & Y. Benno (1999). Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries. FEMS Microbiology and Ecology, 29, 159-169.

Tajima, K., S. Arai, K. Ogata, T. Nagamine, H. Matsui, M. Nakamura, R.I. Aminov & Y. Benno (2000). Rumen bacterial community transition during adaptation to high-grain diet. Anaerobe, 6, 273-284.

Tajima, K., T. Nagamine, H. Matsui, M. Nakamura & R.I. Aminov (2001b). Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. FEMS Microbiolology Letters, 200, 67-72.

Tokura, M., I. Chagan, K. Ushida & Y. Kojima (1999). Phylogenetic study of methanogens associated with rumen ciliates. Current Microbiology, 39, 123-128.

Towne, G. & T.G. Nagaraja (1990). Omasal ciliated protozoa in cattle, bison, and sheep. Applied and Environmental Microbiology, 56, 409-412.

Towne, G., T.G. Nagaraja, R.T. Brandt, Jr. & K.E. Kemp (1990). Dynamics of ruminal ciliated protozoa in feedlot cattle. Applied and Environmental Microbiology, 56, 3174-3178.

Ueda, K., A. Ferlay, J. Chabrot, J.J. Loor, Y. Chilliard & M. Doreau (2003). Effect of linseed oil supplementation on ruminal digestion in dairy cows fed diets with different forage:concentrate. Journal of Dairy Science, 86, 3999-4007.

Van Soest, P.J. (1994). Nutritional Ecology of the Ruminant. 2nd ed., Cornell Univ. Press, Ithaca, NY.

Volden, H. (1999). Effects of level of feeding and ruminally undegraded protein on ruminal bacterial protein synthesis, escape of dietary protein, intestinal amino acid profile, and performance of dairy cows. Journal of Animal Science, 77, 1905-1918.

Volden, H., L.T. Mydland & O.M. Harstad (1999). Chemical composition of protozoal and bacterial fractions isolated from ruminal contents of dairy cows fed diets differing in nitrogen supplementation. Acta Agriculturæ Scandinavica. Section A-Animal Science, 49, 235-244.

von Wintzingerode, F., U.B. Göbel & E. Stackebrandt (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews, 21, 213-229.

Wallace, R.J., R. Onodera & M.A. Cotta (1997). Metabolism of nitrogen-containing compounds. In: Hobson, P.N. & C.S. Stewart (eds) The Rumen Microbial Ecosystem. Chapman and Hall, New York, NY, pp. 283-328.

Weimer, P.J., G.C. Waghorn, C.L. Odt & D.R. Mertens (1999). Effect of diet on populations of three species of ruminal cellulolytic bacteria in lactating dairy cows. Journal of Dairy Science, 82, 122-134.

Weller, R.A. & A.F. Pilgrim (1974). Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. British Journal of Nutrition, 32, 341-351.

Wells, J.E. & J.B. Russell (1996). Why do many ruminal bacteria die and lyse so quickly? Journal of Dairy Science, 79, 1487-1495.

Wells, J.E., J.B. Russell, Y. Shi & P.J. Weimer (1995). Cellodextrin efflux by the cellulolytic ruminal bacterium fibrobacter succinogenes and its potential role in the growth of nonadherent bacteria. Applied and Environmental Microbiology, 61, 1757-1762.

Whitford, M.F., R.J. Forster, C.E. Beard, J. Gong & R.M. Teather (1998). Phylogenic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes. Anaerobe, 4, 153-163.

Williams, A.G. & G.S. Coleman (1997). The rumen protozoa. In: Hobson, P.N. & C.S. Stewart (eds) The Rumen Microbial Ecosystem. Blackie Academic & Professional, New York, pp. 73-139.

Williams, A.G. & G.S. Coleman (1992). The Rumen Protozoa. Springer-Verlag, New York.

Williams, A.G. & S.E. Withers (1993). Changes in the rumen microbial population and its activities during the refaunation period after the reintroduction of ciliate protozoa into the rumen of defaunated sheep. Canadian Journal of Microbiology, 39, 61-69.

Williams, A.G., S.E. Withers & N.H. Strachan (1989). Postprandial variations in the activity of polysaccharide-degrading enzymes in microbial populations from the digesta solids and liquor fractions of rumen contents. Journal of Applied Bacteriology, 66, 15-26.

Wolin, M.J., T.L. Miller & C.S. Stewart (1997). Microbe-microbe interactions. In: Hobson, P.N. & C.S. Stewart (eds) The Rumen Microbial Ecosystem. Blackie Academic & Professional, New York, NY, pp. 467-491.

Wright, A.-D.G. & C. Pimm (2003). Improved strategy for presumptive identification of methanogens using 16S riboprinting. Journal of Microbiological Methods, 55, 337-349.

Wright, A.-D.G., A.J. Williams, B. Winder, C.T. Christopherson, S.L. Rodgers & K.D. Smith (2004). Molecular diversity of rumen methanogens from sheep in western Australia. Applied and Environmental Microbiology, 70, 1263-1270.

Yu, Z. & M. Morrison (2004a). Comparisons of different hypervariable regions of *rrs* genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Applied and Environmental Microbiology, 70, 4800-4806.

Yu, Z. & M. Morrison (2004b). Improved extraction of PCR-quality community DNA from digesta and fecal samples. BioTechniques, 36, 808-812.

Yu, Z., M.A. Yu & M. Morrison (2006). Improved serial analysis of V1 ribosomal sequence tags (SARST-V1) provides a rapid, comprehensive, sequence-based characterization of bacterial diversity and community composition. Environmental Microbiology, in press.

Zatkovic, B., V. Molnarova, V. Kmet, P. Javorsky & P. Pristas (2000). Diversity of DNA sequences among restriction endonucleases producing *Selenomonas ruminantium* isolates detected by enterobacterial repetitive intergenic consensus based polymerase chain reaction (ERIC-PCR). Anaerobe, 6, 299-304.

Ziemer, C.J., R. Sharp, M.D. Stern, M.A. Cotta, T.R. Whitehead & D.A. Stahl (2000). Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. Environmental Microbiology, 2, 632-643.

Zoetendal, E.G., C.T. Collier, S. Koike, R.I. Mackie & H.R. Gaskins (2004). Molecular ecological analysis of the gastrointestinal microbiota: A review. Journal of Nutrition, 134, 465-472.

The role of thermodynamics in the control of ruminal fermentation

E.M. Ungerfeld and R.A. Kohn
Animal and Avian Sciences, University of Maryland, College Park, MD USA 20742

Abstract

Chemical reactions are controlled either thermodynamically or kinetically. With kinetic control, the profile of products formed depends on the substrate concentrations and enzyme activities which control the rates of synthesis for competing pathways. Thermodynamic control occurs when reactants are sufficiently limited relative to products so that the reactions cannot proceed according to the Second Law of Thermodynamics. Under these circumstances, thermodynamics control which pathway branches are available and the final concentration of products. In the rumen, glucose is readily fermented to end products including volatile fatty acids and gases. These end products are removed from the rumen slowly, suggesting that thermodynamics may affect ruminal metabolism. Our calculations show that synthesis of acetate from hydrogen and carbon dioxide is thermodynamically infeasible in the normal rumen. Consistent with these calculations, attempts to increase acetate production by introducing acetogens into the rumen have not been fruitful unless methanogenesis was inhibited or hydrogen pressure was elevated. Addition of intermediates of propionate or butyrate synthesis increased these end products, but also increased acetate production, suggesting that intermediates may increase reaction rates in multiple directions when thermodynamically feasible. Rates of interconversion of major volatile fatty acids measured using isotopes were similar in opposing directions further suggesting that thermodynamics could play an important role in determining profiles of volatile fatty acids. Finally, a novel approach for estimating energy used for ATP production is demonstrated based on existing data and thermodynamic calculations. Although there have been no studies in which all necessary measurements were made to directly determine free energy of fermentation for different reaction pathways, preliminary research from meta-analysis with incomplete data suggests that thermodynamics may play a major role in the control of ruminal metabolic pathways.

Keywords: ruminal fermentation, Second Law of Thermodynamics, metabolic regulation

Introduction

Ruminal fermentation of hexoses results in the production of volatile fatty acids (VFA), namely acetate, propionate and butyrate, and release of the gases carbon dioxide (CO_2) and methane (CH_4). The partition of glucose C among the different VFA is affected by the diet (McDonald *et al*., 1995), and has important consequences for ruminant production and the environment. An understanding of the control of the VFA profile could enable a more logical approach to manipulate the ruminal ecosystem to enhance its beneficial aspects while reducing its negative outcomes. Thermodynamic laws apply to every process in the universe where a large number of particles is involved. The objective of this research is to investigate the possible role of the Second Law of Thermodynamics in controlling ruminal fermentation and the VFA profile.

The profile of VFA formed in the rumen has both economic and environmental consequences for several reasons. The VFA profile can affect the efficiency of energy utilization, composition of animal products (e.g. milk components), and the profile and amount of gases released to the atmosphere. Absorbed VFA are generally the ruminant's main energy substrate, but the fermentation co-products, gases and heat, are released and not used by the host animal. Methane is an energy loss because the reducing equivalents used for producing it could be used by the animal to generate ATP if they were incorporated into useful energy sources.

The stoichiometry of production of the main VFA determines the amounts of CO_2 and CH_4 associated with the formation of acetate, propionate and butyrate. Acetate formation releases two moles of CO_2 and four moles of H_2 per mole of glucose fermented (Figure 1). Four moles of H_2 can then be used by methanogens to reduce one mole of CO_2 to CH_4. Therefore, acetate formation results in the production of one mole of CO_2 and one mole of CH_4 per mole of glucose fermented. Similarly, butyrate production results in 1.5 moles of CO_2 and 0.5 moles of CH_4 per mole of glucose fermented. Propionate formation, on the other hand, does not result in a net production of CO_2 and requires a net input of reducing equivalents, resulting in a decrease in CH_4 production. Due to these differences in the stoichiometries of CH_4 production associated with each VFA, the proportions of the energy in fermented glucose retained as acetate, propionate and butyrate are 0.62, 1.09[1] and 0.78. Propionate formation conserves more of the energy present in fermented glucose in a useful form for the animal (Ørskov and Ryle, 1990).

Part of the energy released by fermentation is used for microbial ATP formation, while the rest dissipates as heat. For animals in thermoneutrality or above, heat production is an energy loss. However, this energy loss is what drives the fermentation process forward, as an overall negative Gibbs energy change ($\Delta G < 0$) is necessary for the process to be spontaneous. Microbial ATP produced during fermentation is used to fuel otherwise non-spontaneous anabolic processes like protein and fatty acid synthesis. Microbial protein synthesized in the rumen is generally the main source of amino acids for ruminants (Wallace *et al.*, 1997). Maximization of microbial protein production allows the use of cheap non-protein N sources in ruminant diets, increases N retention and decreases N voided into the environment (Clark and Davies, 1983).

Thermodynamics can be applied to examine the partition of energy released from fermentation into microbial ATP and heat (Kohn and Boston, 2000). Through substrate level phosphorylation, acetate and butyrate production generate four and two moles of ATP per mole of fermented glucose, respectively (Russell, 2002). Propionate formation by the randomizing (succinate) pathway is associated with ATP production by electron transport-linked phosphorylation in the reduction of fumarate to succinate. However, the stoichiometry of ATP production has not been clearly established. Experiments with membrane preparations of the ruminal bacterium *Vibrio succinogenes* initially yielded between 0.15 and 0.5 moles of ATP produced per mole of fumarate reduced (Reddy and Peck, 1978). However, later work found a value of 0.94 (Kröger and Winkler, 1981). Electron transport-linked phosphorylation has not been demonstrated for propionate's non-randomizing (acrylate) pathway or for butyrate formation (Russell and Wallace, 1997).

[1] Propionate formation does not result in the production of energy. The value of 1.09 is only the ratio between energy in propionate and energy in glucose and does not take into account the oxidation of reducing equivalents needed to produce propionate.

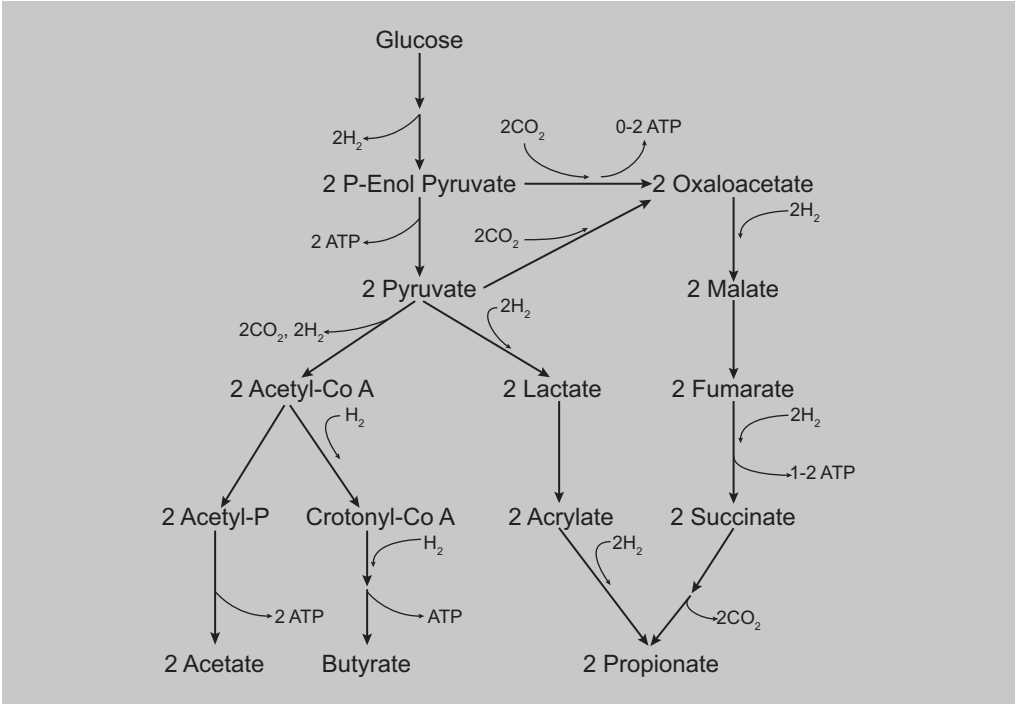

Figure 1. Fermentation pathways in the rumen. Methane is formed from CO_2 and H_2. Although H_2 is not the direct electron donor in the processes depicted, metabolic H is in equilibrium with the H_2 pool. Dihydrogen will be considered the electron donor in our calculations.

The profile of VFA produced in the rumen also has consequences on the host animal metabolism. While propionate formation retains more of the energy fermented in the rumen in useful products, excessive propionate can cause undesirable consequences on production, such as reduced intake (Oba and Allen, 2003), low milk fat content, and soft fat syndrome in lambs (Ørskov and Ryle, 1990).

The profile of VFA formed in the rumen also has environmental consequences. Methane emissions by ruminants are involved in global climate change. At present, CH_4 emissions account for about 18% of total global warming. While CO_2 has a greater effect on global warming than CH_4 at the present time, the latter is increasing at a faster rate (1.1 vs. 0.5% per year) and CH_4 is 50 times more potent than CO_2 on a mass basis as a greenhouse gas (Moss, 1993). Although there are uncertainties in the estimates, approximately 14% of CH_4 emissions may be caused by domestic animals, of which 97% correspond to ruminants (Johnson *et al.*, 1991).

Background

Thermodynamics and kinetics

Chemical reactions are controlled either thermodynamically or kinetically (Chang, 1981). If reactants are sufficiently limited relative to products then the reactions cannot proceed according to the Second Law of Thermodynamics. Under these circumstances, thermodynamics control which pathway branches are available. On the other hand, if the rates of utilization of a substrate control the pathways for product formation where all of the reactions are thermodynamically feasible, the reactions are kinetically controlled. These rates may depend on substrate and/or enzyme concentrations. The difference in behavior between reactions that are controlled kinetically or thermodynamically lies in their response to added substrates, enzymes or products. When kinetics control a process, reaction rates depend on substrate levels and/or enzyme activities but not on product concentrations. When thermodynamics limit reactions, rates depend on substrate and product concentrations but are not affected by enzymes. These differences provide the basis for determining which type of regulation applies, and understanding these differences can elucidate what types of treatments (affecting enzymes, substrates or products) will have an impact.

Consider a system in which key intermediates are found in very low concentrations relative to products. For example, one would not expect to find appreciable concentrations of glucose in the rumen where fermentation occurs rapidly. This expectation results from our understanding that glucose is released from fiber or starch relatively slowly compared to the rate at which it is metabolized to other compounds. The end products of glucose fermentation, volatile fatty acids and gases, are removed relatively slowly. At the very moment a glucose molecule is released into solution it is a candidate for metabolism; whether that molecule becomes acetate, propionate, butyrate or lactate would depend on the speed of the respective reactions or at least on how quickly the reactions can remove the molecule from solution. If one or more end products build up, however, the VFA may inhibit the process from occurring at all based on the Second Law of Thermodynamics. For example, high concentrations of acetate may shift the metabolism toward propionate. The Second Law of Thermodynamics requires that free energy (G) be released for a process to occur spontaneously. If the concentration of precursor becomes small enough relative to product the forward reaction will not proceed. In this case, the precursor may be used to produce a different product, and there may be a shift from one VFA or gas to another. Thermodynamics is a likely means of control for ruminal fermentation for several reasons. For fermentation in the rumen, the substrate (glucose) is found in low concentrations relative to products (VFA and gases), and the microbial population grows and adapts to minimize the potential for enzyme-limiting conditions.

The Second Law of Thermodynamics

The Second Law of Thermodynamics states that the entropy of the universe increases in an irreversible process or remains unchanged in a reversible process (Chang, 1981). Entropy is defined as "randomness" and a reversible process is defined as an infinitely slow one with an infinite number of steps. In fact, most reactions are irreversible and so the entropy of the universe

increases or heat is released from the system to the surroundings. Stated mathematically, the second law is as follows:

$$dS_u \geq 0 \quad (1)$$

where dS_u represents the change in entropy of the universe. This change in entropy can be divided between that of any given system (dS_{system}) and the surroundings of the system ($dS_{surroundings}$); therefore:

$$dS_u = dS_{system} + dS_{surroundings} \quad (2)$$

In a system that is imperfect, heat is generated while work is done. That heat is dissipated from the system to the surroundings. The system can be defined to include all changes in chemical composition of products and reactants. Thus only heat loss from the system needs to be considered as a loss to the surroundings. This heat loss ($-dH_{system}$) must equal the change in entropy of the system when corrected for temperature in degrees Kelvin (T).

$$dS_{surroundings} = -dH_{system} / T \quad (3)$$

By substituting the right hand side of equation 3 into equation 2, a new definition of the change in entropy of the universe is derived:

$$dS_u = dS_{system} - dH_{system} / T \quad (4)$$

Substituting the right hand side of equation 4 into equation 1 reminds us that the change in entropy of the universe must be greater or equal to 0.

$$0 \leq dS_{system} - dH_{system} / T \quad (5)$$

Multiplying both sides by (-T) provides the relation:

$$0 \geq dH_{system} - TdS_{system} \quad (6)$$

This negative value is the definition of the Change in Gibbs Free Energy (ΔG). Thus, ΔG of a system is negative when heat is lost from the system to the surroundings. The more inefficient the system is in using available energy, or the more heat is lost, the more negative the ΔG must be. This relationship will be important to understanding shifts in ruminal metabolism associated with lower efficiency of fermentation and greater heat increment.

Integration of the Second Law of Thermodynamics with the Ideal Gas Law provides a mathematical relationship that is especially useful to chemistry and biology. Under constant pressure, the change in free energy of a reaction is a function of the activity of the products and reactants.

$$\Delta G = \Delta G^\circ + RT \ln \{ [Products] / ([Reactants]) \} \quad (7)$$

where [x] represents activity of x (or pressure in atmospheres of x for ideal gases and molarity of x for ideal solutes), R is the gas constant and equals 8.314 J K^{-1} mol^{-1}, T represents the temperature in degrees Kelvin, and ΔG° represents the change in free energy for the reaction under standard conditions. These conditions are 278.15 K, one unit of activity for all solutes and one atmosphere of pressure for all gases. If a reaction goes to equilibrium, the ΔG becomes zero. Therefore, the ΔG° can be determined by measuring the concentration of products and reactants at equilibrium. Once this value is determined, it can be used with other similar values to determine the ΔG° of other reactions contained within these reactions. The amount of free energy of a material is intrinsic to that material. Furthermore, once the ΔG° is determined for potential reactants and products, the equilibrium constant for those reactions can be easily calculated. Setting ΔG to 0 in equation 7 represents equilibrium. Using table values for the ΔG° enables determination of the equilibrium constant (K_{eq}) as follows:

$$K_{eq} = e^{-\Delta G°/RT} \quad (8)$$

All catalyzed reactions are bi-directional

All catalyzed reactions are bidirectional and the catalyst acts to facilitate the reactions in both directions. Theoretically, it can be stated that catalysts reduce activation energy of reactions, in forward and reverse directions, but they do not change the ΔG of reactions. Or stated another way, a catalyst changes the rate of forward and reverse reactions proportionally, so that equilibrium is not affected, even while the system moves toward equilibrium at a faster rate.

A simple proof of the concept that a catalyst must always accelerate both forward and reverse reactions can be provided by the following example of a perpetual motion machine (Chang, 1981). Imagine a cylinder fitted with a moveable piston in which two moles of gas A can be converted to a single mole of gas B using a solid catalyst contained at the bottom of the cylinder. In this imaginary system, the impossible occurs and the catalyst only accelerates the forward reaction, 2A to B and not the reverse. When the reaction proceeds, the gas volume would shrink, and the piston would lower. You could arrange the catalyst such that as the piston lowers it would close the container for the catalyst and make it unavailable to the gases. Then when gas A is converted to B, the catalyst would be unavailable to the gases and the forward reaction (conversion of 2A to B) would cease. The reverse reaction would continue independent of catalyst causing the piston to rise and making the catalyst available to the gas A again. The piston would rise and lower again and again without expending energy. Thus, we cannot accept the possibility of a catalyst only working in one direction unless we can accept the possibility of a perpetual motion machine.

If a system exists to make propionate or acetate from pyruvate, that system must allow the interconversion of propionate to acetate and the reverse. If propionate production is favored, acetate will be converted to propionate along with new glucose in the system. If there is equilibrium between acetate and propionate, the conversion rates from one to the other will approximate the conversion rates back again. Interconversion of VFA has indeed been shown to occur in many instances, and we will come back to it later.

Example calculations

It is well understood that H_2 is used by methanogens to produce CH_4 which is thought to be a waste product of metabolism. If this H_2 could be used instead to generate acetate from CO_2 through a process called reductive acetogenesis, greater energy could be preserved for production. What is the feasibility of methanogenesis or reductive acetogenesis in the rumen under typical conditions? Kohn and Boston (2000) demonstrated the calculations necessary to answer this type of question. The balanced reactions of interest in this case are:

$$CO_2(aq) + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$$

$$2\ CO_2(aq) + 4\ H_2 \rightarrow C_2H_3O_2^- + H^+ + 2\ H_2O$$

If the total gas pressure in the rumen approximates 1.01325×10^5 Pa (1 atm), and CO_2 comprises 70% of that pressure the pCO_2 would be 7.09×10^4 Pa (0.7 atm). Multiplied by the solubility constant (Segel, 1976) for this ionic strength, temperature and pressure (2.26×10^{-7} mol / Pa), this pCO_2 would provide 0.016 mol / L of dissolved (aq) CO_2 at equilibrium. The H_2 concentration can be calculated using the Nernst equation from the reducing potential and pH (Segel, 1976).

$$\Delta E = \Delta E° + (R\ T / n\ F)\ \ln\ (\ [H^+]^2 / [H_2]_g\ ) \qquad (11)$$

where ΔE is the reducing potential in volts measured using an hydrogen electrode, $\Delta E°$ is the change in reducing potential for the reaction under standard conditions which is equal to zero for the H^+ to H_2 half reaction, R is the gas constant (8.3145 J K^{-1} mol^{-1}), T is the temperature in K, n is the number of moles reduced (2), and F is Faraday's constant (9.6487×10^4 C / mol) which converts ΔE from ΔG. The solution for $[H_2]_g$ in this case yields the concentrations under equilibrium conditions for the H_2 half reaction. Typical ruminal conditions are E = -0.315 V and pH = 6.5 (Barry *et al.*, 1977). Thus, $[H_2]_g$ would be 162 Pa (1.6×10^{-3} atm). For this example, let us assume that the partial pressure of CH_4 is 3.04×10^4 Pa (0.3 atm). Multiplied by its solubility constant (1.97×10^{-10} mol / Pa; Fogg and Gerrard, 1985) yields a concentration of 6×10^{-6} mol / L. The molar mass of water is 18 g/mol with a density of approximately 1000 g per L. Therefore, the molarity of pure water is 55.6 mol / L. Assuming 10% dry matter of the ruminal solvent yields a molarity of approximately 50 mol / L for rumen liquid. Typical ruminal conditions allow for at least 0.05 mol / L of acetate. Thus the concentrations of all products and reactants for a particular set of ruminal conditions have been defined.

Table 1 shows key thermodynamic data under standard conditions for these reactants and products as well as some other important ruminal metabolites. These values represent the free energy of formation ($\Delta G_f°$) and enthalpy of formation ($\Delta H_f°$) of the metabolites from the elements (e.g. H_2, O_2, graphite). Free energy under standard conditions and concentrations ($\Delta G°$) can be determined from these tabular values for each reaction of interest (Chang, 1981).

$$\Delta G° = \Delta G°_f \text{ of products} - \Delta G°_f \text{ reactants} \qquad (12)$$

Table 1. Standard free energy of formation ($\Delta G°_f$) and enthalpy of formation (ΔH_f) in kJ mol^{-1} of key rumen metabolites at 298.15 K and 1.01325 x 10^5 Pa (1 atm).

Metabolite	$\Delta G°_f$	ΔH_f
Acetate (aq)[1]	-376.89	-486
Acetoacetate (aq)	-493.70	not available
Acrylate (aq)	-286.20	-383
Ammonium (aq)	-79.50	-133
Butyrate (aq)	-372.04	-536
Carbon Dioxide (aq)	-386.23	-413
Crotonate (aq)	-275.70	-83
Fumarate (aq)	-651	-777
Glucose (aq)	-916.97	-1264
Hydrogen (aq)	0	0
Hydrogen Sulfide (g)[1]	-27.87	-20
β–hydroxybutyrate (aq)	-506.30	not available
Lactate (aq)	-516.70	-687
Malate (aq)	-845.10	-843
Methane (g)	-50.3	-74
Nitrate (aq)	-110.50	-207
Oxaloacetate (aq)	-797.20	-793
Propionate (aq)	-381.875	-507
Protons (aq)	0	0
Pyruvate (aq)	-474.60	-596
Succinate (aq)	-744	-909
Sulfate (aq)	-742.00	-907
Water (l)[1]	-237.19	-286
Data are *not* adjusted to pH 7 and are from Long (1961), Thauer *et al*. (1977) and Chang (1981). Standard conditions are 1 M concentration of each soluble reactant and product, 1.01325 x 10^5 Pa (1 atm) of all gases, and 298.15K.		
[1] aq = aqueous; g = gas; l = liquid.		

Adjustment to each $\Delta G_f°$ for temperature can be made using a transformation of the van't Hoff equation (Chang, 1981) and enthalpy of formation, $\Delta H_f°$ where T_1 and T_2 are the initial and final temperatures respectively, and $\Delta G°_{T_1}$ and $\Delta G°_{T_2}$ are the respective standard free energy values:

$$\Delta G°_{T_2} = T_2 / T_1[\Delta G°_{T_1} - \Delta H°(T_2 - T_1) / T_2] \quad (13)$$

Table 2 shows the resulting standard change in free energy ($\Delta G°$) calculated for several reactions important to ruminal metabolism under standard conditions and adjusted for 312 K.

Table 2. Estimated energetics of different ruminal redox processes without consideration of ATP changes.

Reduction	[acceptor] (mM)	ΔG° (kJ)	ΔG (kJ)	ΔG (kJ/2H)
$2\ CO_2 + 4\ H_2 \rightarrow CH_3COO^- + H^+ + 2\ H_2O$	0.016	-71.6	-8.8	-2.2
$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$	0.016	-134.0	-67.4	-16.9
$SO_4^{2-} + 4\ H_2 + H^+ \rightarrow HS^- + 4\ H_2O$	0.07 [1]	-234	-84.4	-21.1
$NO_2^- + 3\ H_2 + 2\ H^+ \rightarrow NH_4^+ + 2\ H_2O$	8 [2]	-519	-371	-124
$NO_3^- + H_2 \rightarrow NO_2^- + H_2O$	15 [2]	-161	-130	-130
$Pyruvate^- + H_2 \rightarrow lactate^-$	10^{-6}	-39.8	-5.2	-5.2
Acetoacetyl-CoA + H_2 → β-hydroxybutyryl-CoA	10^{-6}	-34.1	-17.4	-17.4
$Oxaloacetate^{2-} + H_2 \rightarrow malate^{2-}$	10^{-6}	-47.8	-31.1	-31.1
Crotonyl-CoA + H_2 → butyryl-CoA	10^{-6}	-80.7	-64.0	-64.0
$Acrylate^- + H_2 \rightarrow propionate^-$	10^{-6}	-94.3	-51.3	-51.3
$Fumarate^{2-} + H_2 \rightarrow succinate^{2-}$	10^{-6}	-84.0	-63.6	-63.6

ΔG° calculated from ΔG°_f and adjusted to 312 K using the van't Hoff equation as described in Section 2.4.

H_2 does not need to be the electron donor in each process, but ultimately metabolic H is in equilibrium with H_2 and is transferred among microbial species in this form, so H_2 is considered the ultimate electron donor here.

Other conditions: H_2 = 162 Pa (1.6×10^{-3} atm), pH = 6.5; $[H_2O]$ = 50 M; $[succinate^{2-}] = 4 \times 10^{-6}$ M; $[malate^{2-}]$ = [β-hydroxybutyryl-CoA] = [butyryl-CoA]= 10^{-6} M; $[acetate^-]$ = 70 mM; $[propionate^-]$ = 25 mM; $[butyrate^-]$ = 15 mM; $[lactate^-]$ = 1mM; $[NH_4^+]$ = 11 mM (20 mg/dL); $[HS^-]$ = 0.14 mM.

[1] Control treatments in Alves de Oliveira *et al*. (1997).

[2] When feeding high NO_3^- grass (Nakamura *et al*., 1975).

Now it is possible to use equation 7 to determine the ΔG of methanogenesis and acetogenesis under the ruminal conditions that were just described. For methanogenesis,

$$\Delta G = \Delta G^\circ + RT \ln\{ [CH_4]_{aq} [H_2O]^2 / ([CO_2]_{aq} [H_2]_g^4) \} \quad (14)$$

$$\Delta G = -134.0 + .008314 \times 312 \ln \{ (6 \times 10^{-6})(50^2) / [(0.016)(1.6 \times 10^{-3})^4] \} \quad (15)$$

$$\Delta G = -67.4 \text{ kJ / mol} \quad (16)$$

The ΔG for this reaction is negative, so the reaction is feasible. Now consider the ΔG for the use of H_2 for acetate production under the same ruminal conditions.

$$\Delta G = \Delta G^\circ + RT \ln \{ [C_3H_3O_2^-] [H^+] [H_2O]^2 / ([CO_2]_{aq}^2 [H_2]^4) \} \quad (17)$$

$$\Delta G = -71.6 + .008314 \times 312 \ln \{ 0.050\ (1 \times 10^{-6.5})\ 50^2 / [(0.016)^2(1.6 \times 10^{-3})^4] \} \quad (18)$$

$$\Delta G = -8.8 \text{ kJ / mol} \quad (19)$$

The ΔG is negative so the reaction is feasible under these conditions. However, the production of ATP was not considered for these reactions. In fact, including energy for ATP production would have shifted the equilibrium so that more reducing conditions would be needed for acetogenesis.

Thermodynamics and kinetics of H sinks

Reductive acetogenesis versus methanogenesis

Management of H_2 dynamics in the rumen is the key to develop strategies to control ruminant CH_4 emissions (Joblin, 1999). We have shown in the preceding section that methanogenesis is energetically more favorable than reductive acetogenesis under ruminal conditions (Table 2). In agreement, methanogens have H_2 thresholds 10 to 100-fold lower than reductive acetogens (Cord-Ruwisch *et al.*, 1988; Fievez *et al.*, 1999). Thus, methanogenesis outcompetes reductive acetogenesis in the rumen by keeping H_2 pressure under the H_2 threshold for acetogenesis. Attempts to decrease CH_4 production and induce reductive acetogenesis by adding acetogens to ruminal incubations have largely failed (Nollet *et al.*, 1997; Le Van *et al.*, 1998; López *et al.*, 1999a; Fievez *et al.*, 1999). This can be understood on thermodynamic grounds, because the addition of enzymatic activity in the form of live cells would not alter the ΔG of an endergonic process. Reductive acetogenesis can become dominant when H_2 pressure is artificially elevated, as in co-cultures under H_2/CO_2 (Joblin, 1999) or when CH_4 production is inhibited (Nollet *et al.*, 1997; Le Van *et al.*, 1998). The influence of H_2 pressure on the thermodynamics of methanogenesis, reductive acetogenesis, and, another ruminally important reduction, fumarate hydrogenation to succinate, are shown in Figure 2.

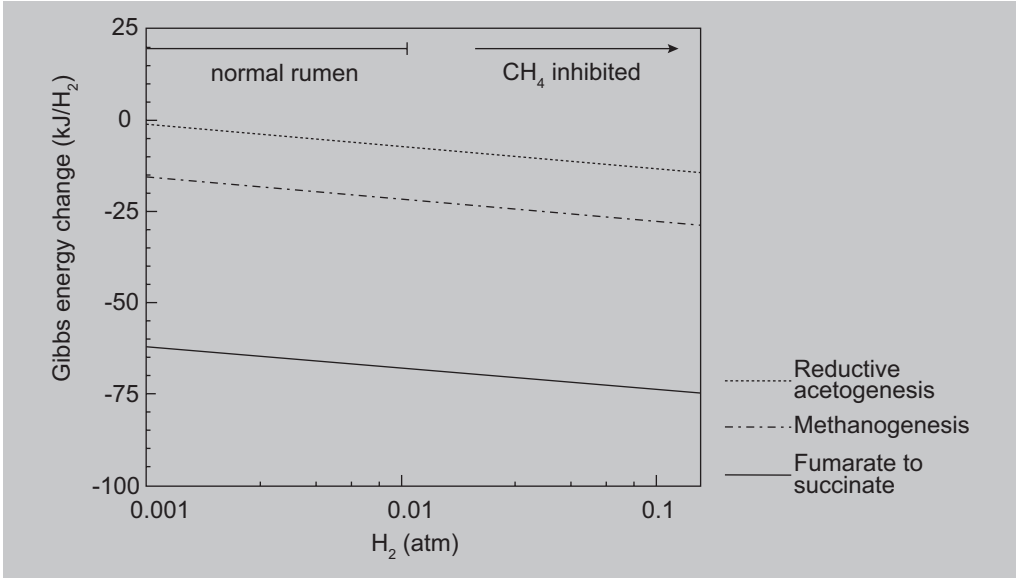

Figure 2. Estimated ΔG per pair of electrons at different H_2 pressures.

At normal ruminal H_2 pressures, the ΔG for reductive acetogenesis is unlikely to drive sufficient ATP synthesis to allow for significant growth[2]. Reductive acetogenesis is minimal in the rumen; acetogens, although present, probably rely on their capacity to utilize a diverse array of substrates other than CO_2 and H_2 as energy sources (Breznak and Kane, 1990; Mackie and Bryant, 1994).

However, reductive acetogenesis constitutes a major electron sink in several animals' hindguts: some species of termites and cockroaches, rodents, some humans, orcas, and pigs (Breznak and Kane, 1990; Mackie and Bryant, 1994), and in the lamb rumen shortly after birth (Morvan *et al*., 1994). Several explanations could be possible for the dominance of reductive acetogenesis or its co-existence with methanogenesis in these environments. Hydrogen gradients exist in methanogenic ecosystems (Boone *et al*., 1989). Acetogens could occupy microniches with higher H_2 availability (Breznak and Kane, 1990), similarly to the symbiotic associations between protozoa and methanogens (Mackie and Bryant, 1994). Also, mixotrophy (the ability to grow on a variety of substrates) could be used advantageously by acetogens to compete with methanogens in some animals' guts (Breznak and Kane, 1990; Mackie and Bryant, 1994).

It is also possible that the host animal is somehow involved in inhibiting methanogenesis to favor reductive acetogenesis, which would increase its energy supply. For example, it was estimated that reductive acetogenesis in the hindgut provided about 1/3 of the energy requirements of the termite *Reticulitermes flavipes*, although the importance of acetogenesis was estimated to be considerable lower in rats and humans (Breznak and Kane, 1990). Phylogenetic analysis of methanogens and their hosts argue for the existence of a host genetic factor affecting the presence of methanogens in the gut (Hackstein, 1997). The numbers of viable methanogens and acetogens were inversely correlated in the termite cecum and in human and rat fecal material (Breznak and Kane, 1990; Fievez *et al*. 1999); conceivably, inhibition of methanogenesis could allow reductive acetogenesis to become the main electron sink in some environments. The presence of circulating antibodies against ruminal methanogens has been reported in sheep (Holloway and Baker, 2002) and perhaps could be related to manipulation of gut fermentation by the host animal. There seem to be important differences in CH_4 emissions among individual grazing sheep (Joblin, 1999) that could possibly be related to antimethanogenic compounds synthesized by the host. However, the addition of cecal contents of a non-methanogenic rabbit or ruminant hindgut mucins to ruminal incubations in vitro did not inhibit methanogenesis (Fievez *et al*., 1999). Bile salts have been shown to inhibit ruminal methanogenesis in vitro, but did not seem to stimulate reductive acetogenesis (Fievez *et al*., 1999).

Fumarate reduction

A major pathway for propionate synthesis in the rumen involves the use of H_2 to reduce fumarate to propionate (Figure 1). Figure 2 shows that fumarate reduction is thermodynamically more favorable than methanogenesis within the range of H_2 pressures of interest. The question then arises as to why ruminal methanogenesis is not outcompeted by propionate formation. It is

[2] A ΔG of -44,000 kJ for ATP synthesis will be considered herein (Kröger, A. & E. Winkler, 1981). However, because acetogens can synthesize ATP coupled to transmembrane electrochemical gradients (Müller & Gottschalk, 1994), the moles of ATP formed per mole of reducing equivalents pair does not need to be an integer number (Voet & Voet, 1995).

important to remember that Figure 2 does not show the effect of ATP generation on ΔG. It is uncertain at present how efficiently the free energy for fumarate reduction can be used for microbial ATP synthesis. It is also unknown how the efficiencies of utilization of ATP for cellular anabolism compare for methanogens and different propionate producers in the rumen.

Fumarate reduction could also be kinetically limited by the availability of H_2 or fumarate. Methanogens had a lower K_m for H_2 than five ruminal fumarate reducers (Asanuma *et al.*, 1999). Attempts have been made to decrease ruminal methanogenesis by adding fumarate to ruminal fermentation. The addition of fumarate would remove kinetic limitations and also improve the energetics of a process that is already more favorable than methanogenesis. A 60% decrease in CH_4 production was initially observed as a result of fumarate addition (Demeyer and Henderickx, 1967). However, the decreases observed in CH_4 production as a result of fumarate addition in seven recent batch culture studies were modest (Figure 3; 37 treatment means; mean decrease = 6%; maximum = 18%; minimum = 0%; sd = 4.4%; Callaway and Martin, 1996; Asanuma *et al.*, 1999; Iwamoto *et al.*, 1999; López *et al.*, 1999b, c; Carro and Ranilla, 2003a; Newbold and Ungerfeld, unpublished):

Although there is a stoichiometrical limitation in the sense that one mole of fumarate reduced to succinate would be expected to decrease CH_4 formation by only 0.25 moles, observed CH_4 decreases in the above seven studies were less than half of what would be expected from the assumption that all added fumarate would be reduced to succinate. The observed decrease in CH_4 production is better explained by the stoichiometry of propionate formed than by added fumarate (Figure 4). In other words, although fumarate has been added as a propionate precursor, only a fraction of added fumarate seemed to be metabolized to propionate in these experiments

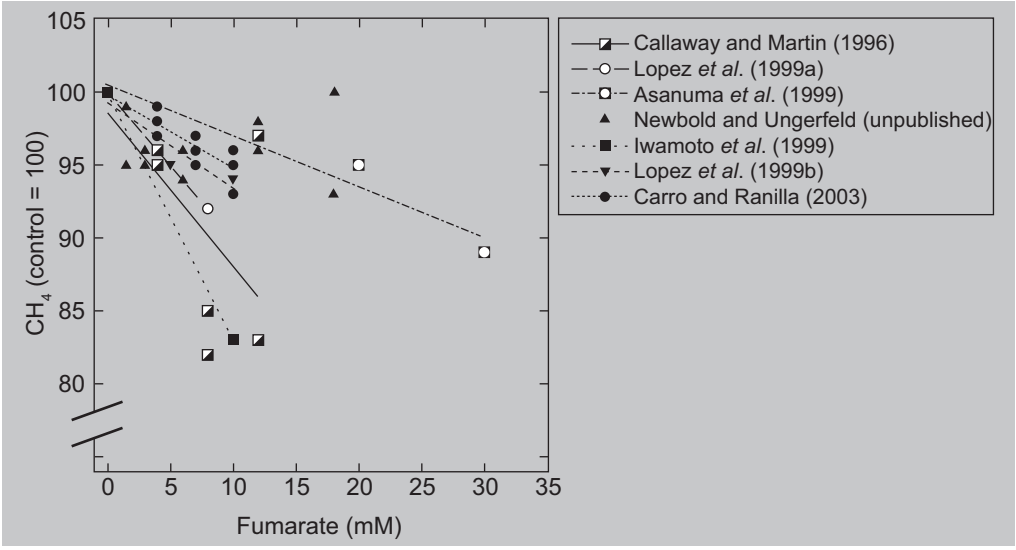

Figure 3. Observed decreases in CH_4 production as a consequence of adding fumarate to ruminal batch cultures.

(fraction of fumarate recovered as propionate: n = 37; mean = 0.58; maximum = 1.33; minimum = -0.68; sd = 0.35). Together with the expected increases in propionate production, the proportion of added fumarate that seemed to be converted to acetate was on average considerable, although it varied amply (recovery of fumarate as acetate: n = 37; mean = 0.35; maximum = 1.47; minimum = 0.076; sd = 0.29). Acetate production from fumarate releases two pairs of reducing equivalents[3]. This process is undesirable because it counterbalances the uptake of reducing equivalents by fumarate. When the reducing equivalents released by acetate formation from fumarate were considered for the H balance, the corresponding decreases in CH_4 production were slightly underestimated (Figure 4), suggesting that fumarate may indirectly stimulate other H sinks. Butyrate production has generally not been affected as a result of fumarate addition to ruminal batch cultures (Callaway and Martin, 1996; Asanuma *et al*., 1999; Iwamoto *et al*., 1999; López *et al*., 1999b, c; Carro and Ranilla, 2003a).

Why a propionate precursor was partly converted to acetate can again be understood on thermodynamic grounds, as the conversion of fumarate into acetate is energetically favorable even at low concentrations of fumarate (Figure 5)[4]. Propionate production from 10 mM fumarate

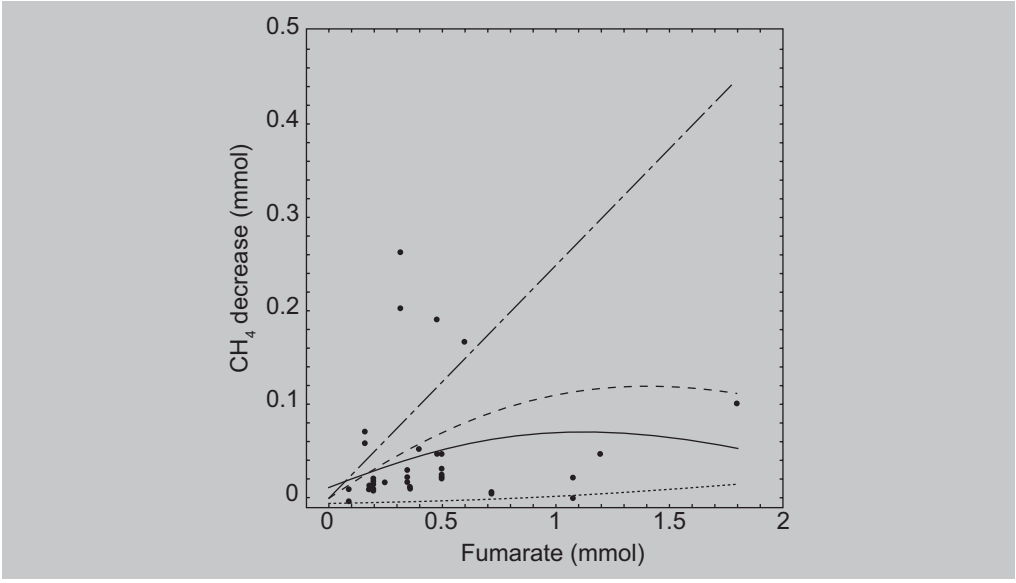

Figure 4. Observed and expected decreases in CH_4 production as a consequence of adding fumarate to ruminal batch cultures. Observed data (•), observed fit (——), predicted from added fumarate (— - —), predicted from extra propionate (– – – –) and predicted from extra propionate and acetate (- - - - -).

[3] If it follows normal ruminal pathways, fumarate would be converted into pyruvate following the reversal of the randomizing (succinate) pathway, and pyruvate would be oxidatively decarboxylated to acetyl-CoA and converted to acetate.

[4] All of these processes would still be thermodynamically favorable if one mole of ATP was synthesized per mole of added fumarate converted to acetate or propionate.

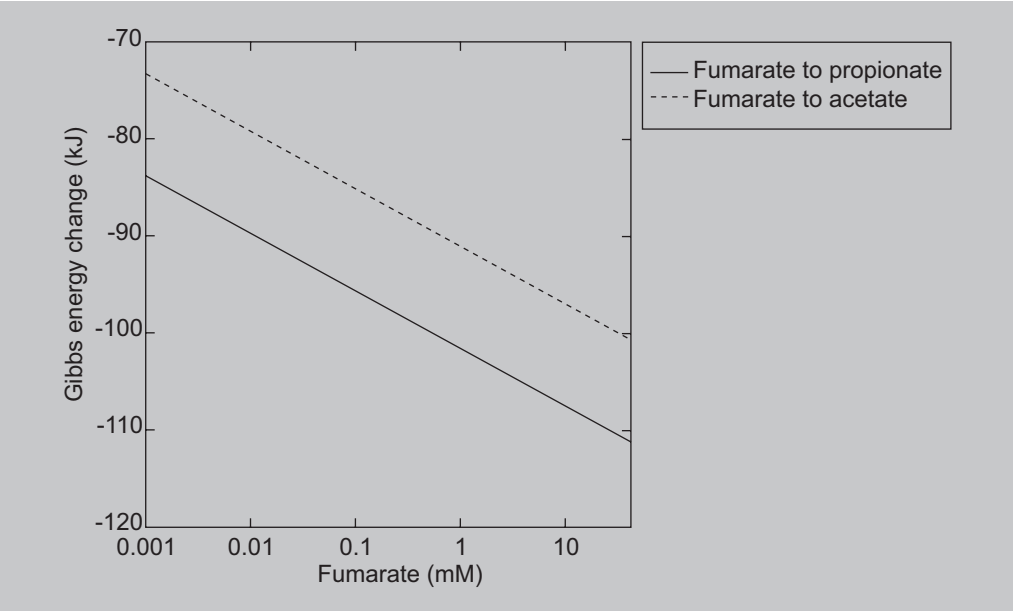

Figure 5. Thermodynamics of fumarate transformations into propionate and acetate.

was increased almost by 7-fold when H_2 pressure was elevated to 1.01 x 10^5 Pa (1 atm), although acetate production was not affected (Schulman and Valentino, 1976).

Thus, if the conversion of fumarate into both acetate and propionate is thermodynamically feasible, the partition of C in added fumarate would be dictated by kinetics. We are not aware of existing kinetic data on the conversion of fumarate into propionate and acetate. With extracellularly added fumarate at relatively high concentrations, V_{max} must be more important than K_m. The kinetics of transport of extracellular fumarate could be a factor influencing the fate of added fumarate. There may be an opportunity to improve the proportion of added fumarate converted into propionate by adding more propionate or succinate producers to the fermentation and enhancing the kinetics of fumarate reduction. Among the bacteria studied by Asanuma *et al.* (1999), *Wolinella succinogenes* had the lowest K_m for H_2 used for fumarate reduction. Perhaps even non-ruminal microorganisms with a high capacity to reduce fumarate could also be used to improve the proportion of fumarate converted to propionate. The capacity of microorganisms to metabolize fumarate to propionate may also possibly be enhanced by genetic manipulation.

In three studies where fumarate was added to ruminal continuous cultures, CH_4 production was decreased by 19% (López *et al.*, 1999c), 28% (Newbold *et al.*, 2001) and 38% (Kolver *et al.*, 2004). In the study by López *et al.* (1999c), there was a 78% recovery of fumarate as propionate, which is higher than in most batch culture studies. This suggests a long term increase in the numbers and/or activities of microbial species that convert fumarate to propionate. However, in the study by Newbold *et al.* (2001), the recovery of fumarate as propionate was only 45%. Adaptation

periods were similar in both studies. López *et al.* (1999c) did not find changes in the magnitude of inhibition of CH_4 production over time or after discontinuing fumarate addition for one week.

In vivo, the inclusion of fumarate in a sheep diet up to 8% DM caused a mild decrease in CH_4 production with a maximum of 9% (Newbold *et al.*, 2001). Therefore, there is no clear evidence from the continuous culture or the in vivo studies of an improvement in the kinetics of conversion of added fumarate to propionate over time, resulting in a stronger inhibition of CH_4 production.

Succinate resulting from fumarate reduction can be converted to propionate in the same cell or transferred to succinate utilizers (Wolin *et al.*, 1997). An improvement in the kinetics of fumarate reduction could result in an increase in succinate concentration, and the question then arises if the conversion of succinate into propionate could become kinetically limited. López *et al.* (1999c) observed a small output of 0.47 mmol/d of succinate when 6.25 mmol/d of sodium fumarate were added to ruminal continuous cultures. On the other hand, Samuelov *et al.* (1999) found that 29.7 mM of added succinate were completely consumed by a mixed ruminal culture, and that more than 90% of it was decarboxylated to propionate, although the time this took is not mentioned.

Malate

Another intermediate of the propionate randomizing (succinate) pathway, malate, is the product of oxaloacetate reduction. Malate is then dehydrated to fumarate in the rumen (Russell, 2002). The reverse reaction, hydration of fumarate to malate, is typically at equilibrium in the Krebs cycle of aerobic organisms (Voet and Voet, 1995). The same as fumarate, malate addition caused a mild inhibition of CH_4 formation in batch cultures (Hino and Asanuma, 2003). The recoveries of malate as propionate (n = 27; mean = 0.52; maximum = 1.05; minimum = -0.15; sd = 0.26) and acetate (n = 27; mean = 0.23; maximum = 0.98; minimum = -0.33; sd = 0.30) in three different ruminal batch culture studies (Martin and Streeter, 1995; Callaway and Martin, 1996; Carro and Ranilla, 2003b) were similar to fumarate. However, on average, the observed decrease in CH_4 production was close to the expected from the stoichiometry of electron withdrawal by the added malate (Figure 6). Malate might have indirectly stimulated other electron sinks or may have benefited cellular anabolism. It has been shown that malate stimulates the uptake and utilization of lactate by *Selenomonas ruminantium*. It has been speculated that malate could be used to overcome a deficiency of oxaloacetate used for gluconeogenesis (Martin, 1998). Whatever the explanation is for the agreement between expected and average observed CH_4 decrease, there was ample variation in the effectiveness of malate at each concentration.

Other electron sinks

The same as fumarate, oxaloacetate is an electron acceptor of propionate's randomizing (succinate) pathway. The reduction of oxaloacetate to malate would have a less favorable ΔG compared to fumarate reduction to succinate (Table 2). In spite of this, as oxaloacetate is upstream of malate or fumarate in propionate's randomizing pathway, its conversion to propionate would incorporate two pairs of reducing equivalents, and the total ΔG change from oxaloacetate to propionate would be

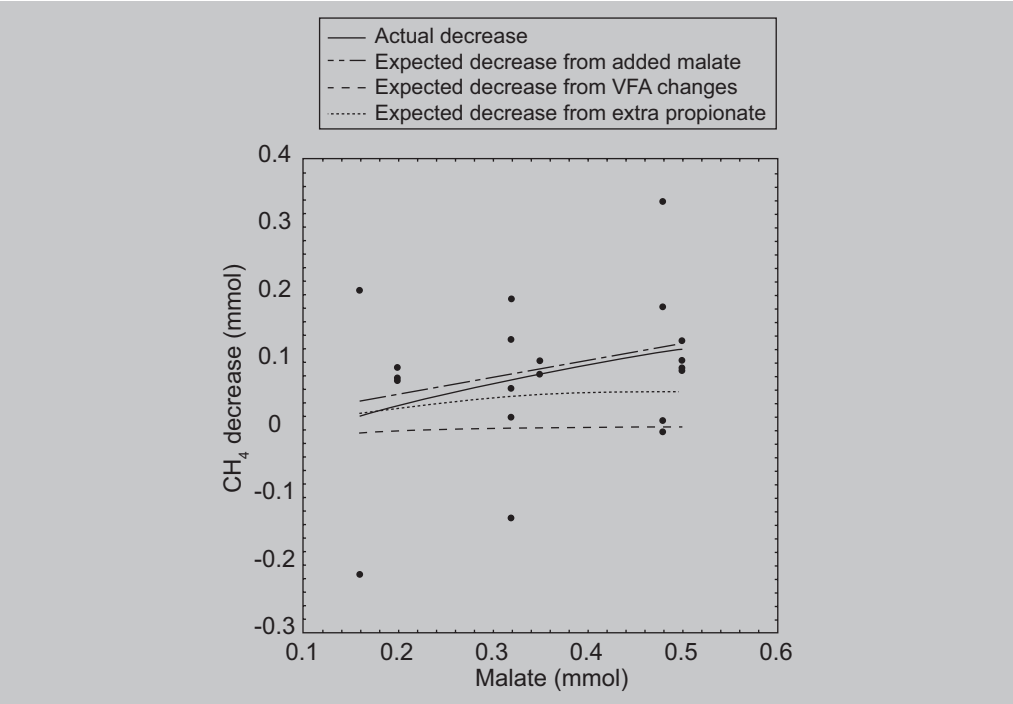

Figure 6. Observed and expected decreases in CH_4 production as a consequence of adding malate to ruminal batch cultures. Data are from Martin and Streeter (1995), Callaway and Martin (1996) and Carro and Ranilla (2003b).
As small increases in butyrate production from added malate have sometimes been found (Callaway & Martin, 1996; Carro & Ranilla, 2003b), these have been taken into account when calculating the expected decrease in CH4 due to changes in VFA production (one mole of malate converted to butyrate would result in the release of one pair of reducing equivalents).

more favorable. However, the addition of oxaloacetate at 6, 12 or 18 mM to ruminal batch cultures resulted in a higher increase in acetate than propionate production, and a slight increase in CH_4 production (Ungerfeld *et al.*, 2003). It can be calculated that, at 6 mM oxaloacetate and typical ruminal concentrations of other reactants and products, the formation of one mole of acetate from oxaloacetate[5] would be energetically favorable even if one mole of ATP was synthesized per mole of oxaloacetate. Therefore, the partition of added oxaloacetate between acetate and propionate must be dictated by kinetics, including the kinetics of oxaloacetate transport into acetate and propionate producing cells.

Deamination of aspartate leads to oxaloacetate (Voet and Voet, 1995). The addition of sodium aspartate to ruminal batch cultures has resulted in an increase in propionate (Callaway and Martin,

[5] Oxaloacetate being decarboxylated to pyruvate, and pyruvate metabolized to acetate by oxidative decarboxylation.

1996; López *et al*., 1999b; Jalč and Čerešňáková, 2001; Jalč *et al*., 2002). Acetate production was increased by aspartate (Callaway and Martin, 1996; López *et al*., 1999b) or not affected (Callaway and Martin, 1996; Jalč and Čerešňáková, 2001). The decrease in CH_4 production was lower than 10% (Callaway and Martin, 1996; López *et al*., 1999b).

Acrylate is an electron acceptor of propionate's non-randomizing pathway (Russell, 2002). The reduction of acrylate to propionate in the rumen would have a ΔG perhaps comparable to fumarate reduction to succinate (Table 2). Although this would be enough to drive ATP synthesis, electron transport-linked phosphorylation has not been demonstrated for this reaction (Thauer *et al*., 1977). The addition of acrylate at 8 mM initial concentration to ruminal batch cultures resulted in a 14% decrease in CH_4 and a 30% increase in propionate. Acetate production did not change, but butyrate production declined 40% (López *et al*., 1999b). In continuous culture, the recovery of acrylate as propionate was slightly lower than fumarate's, resulting in a lower decrease in CH_4 production (Newbold *et al*., 2001).

The reduction of pyruvate to lactate, the first step of propionate's non-randomizing (acrylate) pathway, would seem close to equilibrium in the rumen (Table 2). Pyruvate is the end product of glycolysis and can be converted to acetate, propionate or butyrate (Russell and Wallace, 1997). Pyruvate addition to ruminal batch cultures resulted in 8% less CH_4, 16% more acetate, 10% more propionate and no changes in butyrate (López *et al*., 1999b). The decrease observed in CH_4 production is difficult to explain from the changes in the VFA stoichiometry.

Lactate can be metabolized to propionate both through the randomizing (succinate) or non-randomizing (acrylate) pathway. In either case, there is a net incorporation of one mole of reducing equivalents pair per mole of lactate. In agreement, the addition of H_2 to a N_2 headspace of ruminal batch cultures caused a shift of the fermentation of a lactate substrate from acetate to propionate, and, sometimes, butyrate. The increase in propionate formation, however, seemed to plateau at 19% H_2 in the headspace, and did not increase further at 76% H_2. A shift from acetate to propionate as a consequence of increasing H_2 pressure was also found for fumarate fermentation, but not for pyruvate (Schulman and Valentino, 1976).

Precursors of butyrate formation have also been studied as alternative electron acceptors that could decrease CH_4 formation. The reduction of acetoacetyl-CoA to β-hydroxybutyryl-CoA could be close to equilibrium under ruminal conditions, although intracellular concentrations of these intermediates are largely uncertain (Table 2). Addition of acetoacetate caused a mild decrease in CH_4 in batch cultures, and added acetoacetate seemed to be mainly metabolized to acetate, rather than to butyrate (Ungerfeld *et al*., 2003). The reduction of crotonyl-CoA to butyryl-CoA would be energetically favorable (Table 2). However, ATP synthesis associated to this hydrogenation has not been demonstrated (Thauer *et al*., 1977). A greater proportion of added crotonic acid seemed to be metabolized to butyrate compared to other butyrate precursors; however, the increase in acetate production was still important, and the decrease in CH_4 production was small (Ungerfeld *et al*., 2003).

Biohydrogenation of fatty acids was proposed as an alternative electron sink to methanogenesis. Unsaturated fats and oils decrease CH_4 production, but the reduction in methanogenesis is

greater than what the stoichiometry of biohydrogentation would allow (Nagaraja *et al*., 1997). Studies with pure cultures revealed that ruminal methanogens are very sensitive to long chain, unsaturated fatty acids (Prins *et al*., 1972; Henderson, 1973). Also, some Gram positive bacteria that produce CH_4 precursors are more inhibited by long chain, unsaturated fatty acids than Gram negative bacteria important in propionate formation (Henderson, 1973; Maczulak *et al*., 1981).

In the rumen, NO_3^- is reduced to NO_2^- and then to ammonium ion (NH_4^+), a process that overall captures four moles of reducing equivalents per mole of NO_3^- reduced (Hino and Asanuma, 2003). Both NO_3^- reduction to NO_2^- and NO_2^- reduction to NH_4^+ are energetically very favorable (Table 2); the latter is, however, slower than the former (Iwamoto *et al*., 1999). Thus, although ruminal reduction of NO_3^- present in plants decreases CH_4 production, this benefit is counterbalanced by the accumulation of NO_2^-, which is toxic (Takahashi, 2001).

Sulfate (SO_4^{2-}) reduction can be assimilatory or dissimilatory. Assimilatory SO_4^{2-} reduction incorporates S into S-containing amino acids, while dissimilatory SO_4^{2-} reduction releases hydrogen sulfide (H_2S) as an electron sink resulting from electron transport-linked phosphorylation (Alves de Oliveira *et al*., 1997). At a pH of 6.5, 87% of H_2S would be dissociated to sulfidric anion (HS^-; pK_a = 7.24). Sulfate reduction to HS^- has a favorable ΔG (Table 2). Hydrogen sulfide and HS^- are in equilibrium, and H_2S is released to the rumen gas phase. Gaseous H_2S is not an energy substrate for ruminants and can cause cerebrocortical necrosis (Alves de Oliveira *et al*., 1997).

As microbial biomass is more reduced than feed substrates (Hungate *et al*., 1961), it can be an electron sink itself. Anabolic processes like amination and lipid synthesis incorporate reducing equivalents (Czerkawski, 1986; Voet and Voet, 1995). Consequently, increasing microbial biomass synthesis could help decreasing CH_4 production.

Thermodynamics and kinetics of VFA interconversion

The production of acetate, propionate and butyrate share glycolysis as a common pathway, with pyruvate being the central branching point where the different pathways diverge. Acetate and butyrate formation also share acetyl-CoA as a common intermediate (Russell and Wallace, 1997; Russell, 2002). Kohn and Boston (2000) estimated the free energy changes of each fermentation pathway for typical ruminal conditions and assuming a synthesis of 4 moles of ATP per mole of glucose converted to propionate. After taking into account the estimated ΔG used for microbial ATP synthesis, the proportion of ΔG released as heat was similar for acetate and propionate, and somewhat greater for butyrate production.

The conversion of pyruvate into acetate, propionate or butyrate is a spontaneous process with a negative ΔG. The existence of common intermediates implies thermodynamic interconnectedness among the different end products. To say that the ΔG of the conversion of pyruvate into the different fermentation products is similar is equivalent to saying that the ΔG of interconversion of the fermentation products is close to zero. If that is the case, there would be thermodynamic equilibrium among VFA, and the VFA profile would be thermodynamically controlled. Calculations of ΔG for the interconversion among acetate and propionate and acetate and butyrate

based on arbitrary but typical conditions suggest that the system may operate close to equilibrium for these interconversions (Table 3).

If two given VFA are in equilibrium, then the flows of interconversion between them, adjusted by the corresponding stoichiometrical coefficients, should be equal, as the same enzymes are involved in the forward and reverse transformations. Even if the flows in each direction are not identical, thermodynamics still plays a role in the partitioning of C if the flows in one direction are a significant proportion of the flows in the other direction (i.e., in a system close to equilibrium). For example, if acetate conversion to propionate is four times faster than the reverse, the reverse reaction decreases the net flow by 25% compared to the same reaction without thermodynamic limitations (i.e., when the system is far away from equilibrium).

Interconversion of VFA in the rumen was noticed and reported early on, along with the first measurements of VFA production in vivo (Gray *et al*., 1952, 1960, 1965; Sheppard *et al*., 1959; Van Campen and Matrone, 1960). When a labeled VFA was infused into the rumen continuously or in a single dose in order to calculate its production rates, it was noticed that the label also appeared in other VFA. The ratio between specific activities of the secondary and the primary VFA pool allowed calculating which proportion of the gross production rate was not de novo production but rather originated from a different VFA. Then, gross production rates could be corrected for interconversion, and net production rates calculated. Published observations where labeled VFA were infused can be used to study how close to equilibrium were the VFA in the different systems described. Ratios between opposite interconversion flows between acetate and propionate and acetate and butyrate, calculated from published data, are shown in Table 4.

Fourteen out of 17 ratios of acetate and propionate interconversion flows were within one order of magnitude of unity, which would be equilibrium. Ratios between interconversion flows did not seem to depend on the type of diet ($P = 0.76$; Kruskal-Wallis ANOVA) or animal ($P = 0.29$; Kruskal-Wallis ANOVA). Acetate and butyrate were also generally close to equilibrium (which would be at a ratio of two after adjusting acetate flow by the stoichiometrical coefficient). Fifteen out of the 16 interconversion flows ratios were within one order of magnitude of equilibrium, although there generally was a net flow from acetate towards butyrate. There was a tendency ($P = 0.09$; Kruskal-Wallis ANOVA) for higher ratios of interconversion flows between acetate and butyrate for high concentrate diets, although this could be a trial artifact, as the only two data on

Table 3. Calculated ΔG for interconversions between acetate and propionate and acetate and butyrate[1].

Diet	A[2]	P[3]	B[4]	H_2	CH_4	CO_2	pH	$\Delta G_{A \rightarrow P}$	$\Delta G_{A \rightarrow B}$
	mM	mM	mM	Pa	Pa[5]	Pa[6]		kJ[7]	kJ[8]
High roughage	70	20	12	162	33,437	67,888	6.5	-11.2	4.2
High concentrate	67	49	24	263	25,331	75,994	5.4	-12.5	-2.8

[1]No net ATP synthesis or utilization is assumed at this point; [2]A = acetate; [3]P = propionate; [4] B = butyrate; [5] $[CH_4] = P_{CH4} \times 1.97 \times 10^{-10}$ M/Pa; [6] $[CO_2] = P_{CO2} \times 2.26 \times 10^{-7}$ M/Pa; [7] $\Delta G_{A \rightarrow P} = \Delta G°_{A \rightarrow P} + RT \text{ Ln} ([P] [H_2O]^2 / [A][CO_2][H_2]^3)$; [8] $\Delta G_{A \rightarrow B} = \Delta G°_{A \rightarrow B} + RT \text{ Ln}([B] [H_2O]^2 / [A]^2[H_2]^2[H^+])$. All calculations based on data from Kohn and Boston (2000).

Table 4. Ratios between opposite VFA interconversion flows.

Reference	Animal	Treatment and feeding frequency	$F_{A\rightarrow P}$ / $F_{P\rightarrow A}$ [1]	$F_{A\rightarrow B}$ / $F_{B\rightarrow A}$ [2]
Leng and Leonard (1965)[3]	Sheep	Alfalfa chaff, every h	0.36	1.06
Bergman *et al*. (1965)	Sheep	Pelleted grass, continuous	0.81	2.73
Leng and Brett (1966)[4]	Sheep	Alfalfa chaff, every h	∞	2.13
Leng and Brett (1966)[4]	Sheep	2/3 corn, 1/3 alfalfa chaff, every h	1.00	2.44
Leng and Brett (1966) [4]	Sheep	½ corn, ½ alfalfa chaff, every h	0.00	1.88
Leng and Brett (1966)[4]	Sheep	9/10 wheat straw, 1/10 alfalfa, every h	-	3.00
Weller *et al*. (1967)	Sheep	6/10 alfalfa hay, 4/10 wheat chaff, every 12 h	12.9	34.0
Esdale *et al*. (1969)	Cows	Corn silage, every h	3.46	4.20
Esdale *et al*. (1969)	Cows	Alfalfa hay, every h	2.77	2.22
Marwaha *et al*. (1972)	In vitro	Grass	0.79	2.70
Marwaha *et al*. (1972)	In vitro	Grass + Na sulfate	0.90	1.55
Marwaha *et al*. (1972)	In vitro	Grass + Na thiosulfate	0.69	2.33
Sharp *et al*. (1982)	Steers	84% ground corn, every h	0.32	6.19
Sharp *et al*. (1982)	Steers	84% whole corn, every h	1.65	7.14
Armentano and Young (1983)[4]	Steers	7/10 alfalfa hay, 3/10 corn, continuous	1.50	1.82
Bruce (1988)	Sheep	Dried grass, continuous	1.23	5.04
Seal and Parker (1994)	Steers	Grass pellet, continuous, no infusion	0.83	-
Seal and Parker (1994)	Steers	Grass pellet, continuous, C_3 infusion	0.33	-
Median			0.90	2.57
Lower quartile			0.36	2.00
Upper quartile	n		1.57	4.62
Median roughage	13		0.87	2.70
Median mixed	3		1.00	1.88
Median high-concentrate	2		0.99	6.67

[1] $F_{A\rightarrow P}$ / $F_{P\rightarrow A}$ = ratio between flow of acetate converted to propionate and flow of propionate converted to acetate.

[2] $F_{A\rightarrow B}$ / $F_{B\rightarrow A}$ = ratio between flow of acetate converted to butyrate and flow of butyrate converted to acetate.

[3] Labeling of propionate's C1 would underestimate $F_{P\rightarrow A}$.

[4] Universal labeling of propionate would underestimate $F_{P\rightarrow A}$.

high concentrate are from the same study (Sharp *et al*., 1982). There was no influence of the type of animal on the ratio of interconversion flows between acetate and butyrate ($P = 0.79$; Kruskal-Wallis ANOVA).

Seal and Parker (1994) found similar interconversion flows between acetate and propionate in the rumen of steers. However, when propionate was infused into the rumen, increasing its concentration from 15 to 25 mM, the flow of propionate into acetate became 3.2 fold greater than the flow of acetate into propionate. Acetate production rate decreased 15%. Their results indicate that, at least at the concentration of propionate achieved by the level of propionate infusion used, the ratio between acetate and propionate was thermodynamically regulated.

Some of the published work on VFA interconversion includes data on pH and VFA concentration, although none of the studies found measured gases. Assuming partial pressures of CO_2, CH_4 and H_2 of 7.09 x 10^4 Pa, 3.04 x 10^4 Pa and 162 Pa, respectively (0.7, 0.3 and 1.6 x 10^{-3} atm, respectively; Kohn and Boston, 2000), the ΔG for these interconversions can be estimated, and related to the ratios of measured opposite interconversion flows. The relationship between the ratio of opposite interconversion flows between acetate and propionate (normalized to a logarithm scale) and estimated ΔG is shown in Figure 7.

Although the ratio of interconversion flows was close to unity (zero when log transgormed), the ΔG does not equal 0, perhaps partly because of differences that were not considered in ATP generated between reactions. A negative slope would be expected because a higher free energy change should drive the reaction toward propionate; however the slope estimate did not differ from 0 ($P = 0.31$). One reason we may not see a significant slope is the narrow range in the data. Some limitations of the data available for the current application are lack of direct measurements of gases, and propionate labeling in the first C (Leng and Leonard, 1965) or universally (Leng and Brett, 1966), which would underestimate flow from propionate to acetate.

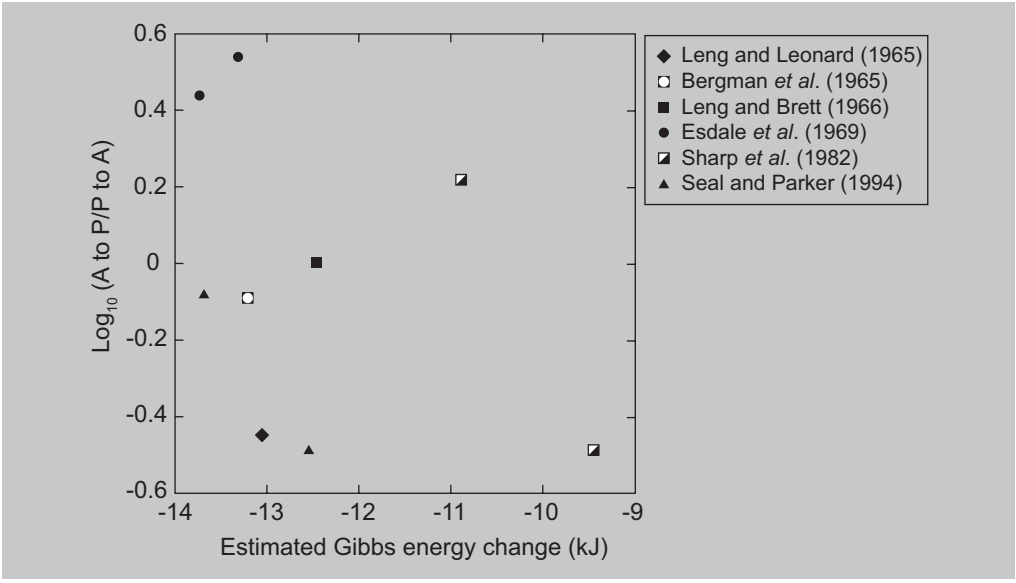

Figure 7. Relationship between the ratio of opposite interconversion flows between acetate and propionate and the calculated free energy change without consideration of ATP changes.

For acetate conversion to butyrate (Figure 8), estimated ΔG was close to 0, which would agree with ratios between opposite interconversion flows close to two (after adjusting for the stoichiometrical coefficients[6]) if there are no differences in ATP generated between pathways, or the differences in ATP are counterbalanced by different efficiencies. There was a tendency (P = 0.10) for a quadratic relationship between this decimal logarithm of the interconversion flows ratio and ΔG; we do not have an explanation for this response at the moment.

In all of the studies used in this analysis, measurements of VFA specific activity were performed in VFA extracellular pools (i.e., in cell-free ruminal fluid). Assuming that the protonated species can freely diffuse across cell envelopes, extracellular and intracellular concentrations of undissociated VFA should be equal, and in equilibrium with the dissociated species both intra and extracellularly (Russell, 1991). Then, intra and extracellular pools of VFA anions would be in equilibrium. At an ideal steady state, measurements of VFA interconversion flows done in the fluid phase would reflect intracellular events. In real, non-steady state systems, however, equilibration may not occur rapidly. Because of the feeding regime employed, the experiment by Weller *et al*. (1967) would have been the farthest away from steady state among the in vivo data (Table 4). Interestingly, the ratios between opposite interconversion flows were not within one order of magnitude of equilibrium in this study. The in vitro batch culture study by Marwaha

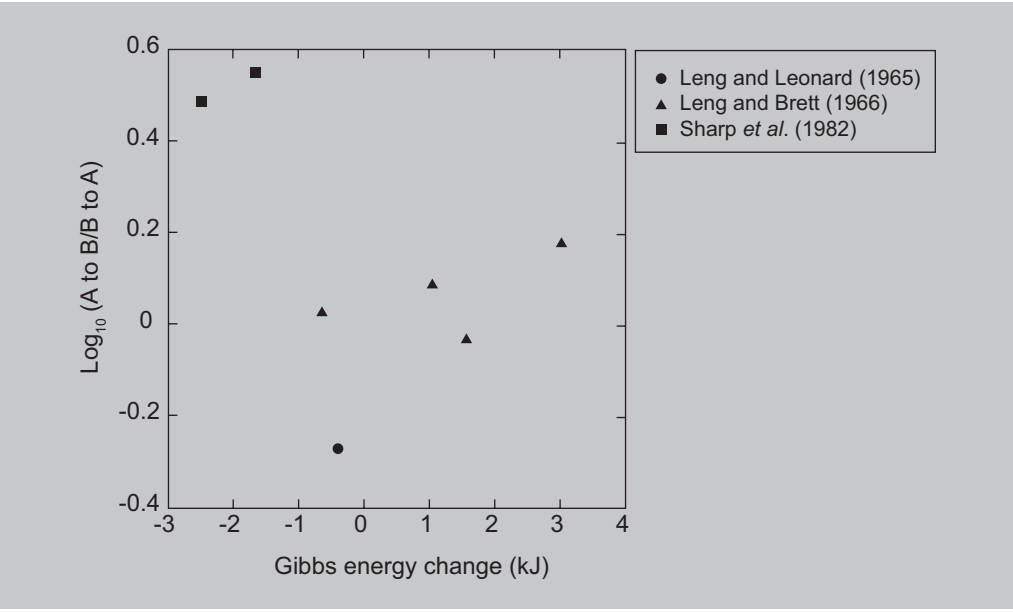

Figure 8. Relationship between the ratio of opposite interconversion flows between acetate and butyrate and the calculated free energy change.

6 In order to normalize the ratio of opposite interconversion flows between acetate and butyrate to a log 10 scale with zero being equilibrium, each ratio was divided by two before log transforming. In this way, a ratio between interconversion flows of two (equilibrium) becomes zero.

et al. (1972) was evidently at non-steady state; however, equilibration between the extracellular labeled VFA pools and the intracellular ones might have occurred after the 24 h period after which measurements were taken.

The stoichiometry of ATP generation during VFA interconversions will now be addressed. As discussed earlier, there is uncertainty in the number of ATP produced in propionate formation by the randomizing (succinate) pathway. Consequently, ATP formed or used during the interconversion between acetate and propionate is also uncertain. If interconversion between acetate and butyrate occurs through the same metabolic pathways by which these VFA are formed, one mole of ATP would be utilized per two moles of acetate converted into one mole of butyrate (since acetate formation from pyruvate yields two ATP while butyrate's yields only one).

The ratios between opposite interconversion flows can be used together with the reactants and products concentrations and pressures to estimate the K_{eq} and free energy available for ATP generation at equilibrium. For example, for the interconversion between acetate and propionate:

$$CH_3COO^- + CO_2 + 3\ H_2 + n\ ADP + n\ P_i \leftrightarrow CH_3CH_2COO^- + (2 + n)\ H_2O + n\ ATP$$

$$V_{A\rightarrow P} = -d[CH_3COO^-] / dt = K_{A\rightarrow P}\ [CH_3COO^-][CO_2]P_{H2}{}^3\ ([ADP][P_i][H^+])^n \quad (20)$$

and

$$V_{P\rightarrow A} = -d[CH_3CH_2COO^-] / dt = K_{P\rightarrow A}\ [CH_3CH_2COO^-][H_2O]^2\ ([ATP][H_2O])^n \quad (21)$$

where $V_{A\rightarrow P}$ and $V_{P\rightarrow A}$ are the velocities for the forward and reverse processes, respectively, $k_{A\rightarrow P}$ and $k_{P\rightarrow A}$ are the corresponding kinetic rate constants, and n represents the moles of ATP generated (or consumed, if negative). The ratio (R) between opposite interconversion flows would then be equal to:

$$R = V_{A\rightarrow P} / V_{P\rightarrow A} = \frac{K_{A\rightarrow P}\ [CH_3COO^-]\ [CO_2]\ P_{H2}{}^3\ ([ADP][P_i][H^+])^n}{K_{P\rightarrow A}\ [CH_3CH_2COO^-]\ [H_2O]^2\ ([ATP][H_2O])^n} \quad (22)$$

Then, as

$$K_{A\rightarrow P} / K_{P\rightarrow A} = K_{eq} \quad (23)$$

$$R = K_{eq} \times Q \quad (24)$$

where Q is defined as the ratio of reactant to product pressures and concentrations adjusted by their corresponding stoichiometrical coefficients. In the calculations that follow [ATP] was assumed to be 1 mmol L^{-1}, [ADP][Pi] was assumed to be 0.002 $mmol^2\ L^{-2}$, and $[H_2O]$ was assumed to be 50 mol L^{-1}, and pH 6.5. Then:

$$K_{eq} = R / Q \quad (25)$$

If we assume that the same proportion of free energy is captured by ATP generation for both acetate and propionate, the ΔG for interconversion will be 0 when in equilibrium. When at equilibrium, R = 1, and Q = K_{eq}^{-1} (from equation 24). The following relationship then holds:

$\Delta G° = -R\ T\ Ln\ (K_{eq})$ (see Equation 8)

This ΔG° represents the interconversion reaction coupled to ATP synthesis ($\Delta G°_{rxn+ATP}$), and can be partitioned between the ΔG° for the uncoupled reaction ($\Delta G°_{rxn}$) and that for ATP synthesis ($\Delta G°_{ATP}$):

$$\Delta G°_{rxn+ATP} = \Delta G°_{rxn} + n\ \Delta G°_{ATP} \quad (26)$$

Solving for n yields the theoretical difference from the interconversion from acetate to propionate. The difference between ΔG° calculated from experimentally measured flows and concentrations, ($\Delta G°_{rxn+ATP}$) as above, and ΔG° calculated from ΔG_f of reactants and products without considering ATP changes ($\Delta G°_{rxn}$), can be attributed, at least partly, to ΔG from ATP utilization or production ($n\Delta G°_{ATP} = \Delta G°_{rxn+ATP} - \Delta G°_{rxn}$). The stoichiometry of ATP utilization or production (n) can then be calculated by dividing this difference ($n\Delta G°_{ATP}$) by a $\Delta G°_{ATP}$ of -20.5 kJ mol^{-1} for ATP synthesis. Standard conditions assume pH = 0, which results in the negative value for ATP synthesis. This value corresponds to $\Delta G°'_{ATP}$ = 31 kJ mol^{-1} at pH 6.5, and under the physiological conditions described for equation 24, ΔG would be 44 kJ mol^{-1}. Although standard conditions are not physiologically possible, the ΔG° can still be used to calculate ΔG under physiological conditions.

Table 5 summarizes the estimates for ATP synthesis for acetate and propionate and acetate and butyrate interconversions based on this calculation. Values in Table 5 can be used to calculate ΔG for VFA interconversion using equation 7. Standard conditions are quite dissimilar to actual conditions, so eyeballing thermodynamic limits from ΔG° is not recommended. For acetate conversion to propionate, the average difference between $\Delta G°_{rxn+ATP}$ and $\Delta G°_{rxn}$ was 12.2 kJ mol^{-1}. This difference may represent differences in heat loss between the two types of fermentation (acetate or propionate) as well as differences in free energy trapped in ATP. If the difference was entirely ascribed to ATP synthesis, there would be enough free energy to synthesize 0.60 moles of ATP per mole of propionate converted to acetate (or, synthesis of one mole of propionate from one mole of acetate would require 0.60 moles of ATP). In other words, assuming each type of fermentation is similar in efficiency of trapping free energy, propionate would generate 0.60 moles less ATP per mole of end product than acetate fermentation. This prediction is consistent with the estimates of ATP generation shown in Figure 1. Converting acetate to acetyl-P would require 1 mole of ATP, but fumarate reduction would generate 0.5 to 1.0 moles of ATP (Reddy and Peck, 1978; Kröger and Winkler, 1981), or reduction through the lactate-acrylate (non-ransomizing) pathway would generate no ATP (Thauer *et al*., 1977). Thus, the observation that 0.60 moles of ATP are used in the interconversion of acetate to propionate is in line with the expected generation of between 0 and 1 moles of ATP for pyruvate conversion to propionate (Figure 1). Once absorbed, propionate is a higher energy compound than acetate, but acetate production may enable microbes to harvest more energy. Acetate fermentation traps additional ATP when methanogens use the H_2 that is produced.

Table 5. ATP stoichiometry for VFA interconversion estimated from interconversion flows and reactant and product concentrations and pressure.

	$A^- + CO_2 + 3H_2 \leftrightarrow P^- + 2H_2O$			$2A^- + 2H_2 + H^+ \leftrightarrow B^- + 2H_2O$		
	$\Delta G°_{rxn +ATP}$ kJ mol^{-1}	$n\Delta G°_{ATP}$ kJ mol^{-1}	ATP[1] mol	$\Delta G°_{rxn +ATP}$ kJ mol^{-1}	$n\Delta G°_{ATP}$ kJ mol^{-1}	ATP[1] mol
Leng and Leonard (1965)	-78.6	10.4	-0.51	-90.1	0.6	-0.03
Bergman *et al*. (1965)	-76.4	12.7	-0.62	-	-	
Leng and Brett (1966)	-76.6	12.5	-0.61	-88.0	2.6	-0.13
Leng and Brett (1966)	-	-		-89.4	1.3	-0.06
Leng and Brett (1966)	-	-		-90.6	0.05	0.00
Leng and Brett (1966)	-	-		-90.8	-0.20	0.01
Esdale *et al*. (1969)	-72.5	16.5	-0.80	-	-	
Esdale *et al*. (1969)	-72.6	16.4	-0.80	-	-	
Sharp *et al*. (1982)	-82.5	6.5	-0.32	-83.4	7.2	-0.35
Sharp *et al*. (1982)	-76.8	12.2	-0.60	-83.9	6.7	-0.33
Seal and Parker (1994)	-75.8	13.2	-0.64	-	-	
Seal and Parker (1994)	-79.4	9.6	-0.47	-	-	
Mean	-76.8	12.2	-.60	-88.0	2.6	-.13
SD	3.2	3.2	.15	3.1	3.1	.15

A^- = acetate, P^- = propionate, B^- = butyrate. The ΔG for interconversion reaction including ATP generation ($\Delta G°_{rxn +ATP}$) was determined from equilibrium constants calculated from measured interconversion flows. [1]Theoretical maximum ATP production from free energy for ATP generation assuming no difference in free energy efficiency between acetate and propionate (ΔG = 0 for interconversion). Calculated as $n = (\Delta G°_{rxn +ATP} - \Delta G°_{rxn}) / \Delta G°_{ATP}$ (equation 26) $\Delta G°_{rxn}$ = -89.0 kJ mol^{-1} for acetate to propionate; $\Delta G°_{rxn}$ = -90.7 kJ mol^{-1} for acetate to butyrate, $\Delta G°_{ATP}$ = -20.5 (Rekharsky *et al*. 1986; not adjusted for pH but adjusted for temperature).

Estimated changes in ATP for acetate and butyrate interconversion were close to zero, but known pathways suggest that conversion of acetate to butyrate should require 0.5 ATP per mole of acetate (see Figure 1). There can be two explanations for this discrepancy. Perhaps, butyrate formation is highly inefficient resulting in considerably more heat than acetate or propionate production. Based on typical ruminal concentrations, Kohn and Boston (2000) also noted a very low efficiency of trapping ATP for butyrate compared to acetate and propionate assuming only 1 ATP generated per butyrate from two acetyl-CoA. Another possibility is that butyrate synthesis generates 2 ATP rather than one in the pathway from acetyl-CoA. Thermodynamics would indicate that the there may be adequate energy for ATP generation in the reduction of crotonyl-CoA to butyryl-CoA, although so far this has not been found experimentally (Thauer, 1977).

We are intrigued by the relative consistency from study to study of the calculated ATP stochiometry shown in Table 4 considering that data were derived using sheep, cattle and in vitro preparations with different diets. This result aligns with others that suggest constancy in the control of the VFA profile, indicating that VFA could be near thermodynamic equilibrium with each other.

Fermentation pathways shift during transition to high-starch diets (Kohn and Boston, 2000). Pathways that capture less free energy are likely to proceed faster, but not be feasible on low amounts of energy (Jou and Llebot, 1990). The lactate-acrylate pathway captures less energy as ATP compared with the succinate pathway. Although similar ΔG for interconversion were obtained across studies and types of diet, more of the ΔG for interconversion between propionate and acetate may be released as heat rather than ATP when fermentation pathways shift on high starch diets.

Conclusions

Under ruminal conditions, reductive acetogenesis seems to be thermodynamically limited, as methanogens decrease H_2 pressure below the reductive acetogenesis threshold. Thermodynamics predict that introducing reductive acetogens into the rumen will not have an effect, and that agrees with experimental results. However, reductive acetogenesis co-exists or even dominates methanogenesis in some animals' hindguts. The comparison of physicochemical and microbiological conditions in these gut environments and the rumen offers a good opportunity to learn what can be done to stimulate reductive acetogenesis in the rumen.

The addition of some propionate and butyrate precursors (e.g. fumarate, malate, crotonate) to ruminal fermentation resulted in reducing equivalents being incorporated into propionate and butyrate. However, part of these metabolic intermediates was also converted to acetate, releasing reducing equivalents. The result was that the decreases in CH_4 production by lowering H_2 available were lower than expected. Calculation of the thermodyamics for these reactions can explain this result.

Thermodynamic calculations for typical ruminal conditions and results from published studies where flows of VFA interconversion were measured suggest that acetate, propionate and butyrate may be close to equilibrium with each other. Even when interconversion flows in both directions differed, opposite flows were generally within one order of magnitude of each other. This would mean that the VFA profiles could be thermodynamically influenced even when acetate and propionate are not at equilibrium, because reverse interconversion flows would be a significant fraction of forward flows. Even though this hypothesis still does not explain the VFA shifts that occur when changing types of diets, this analysis suggests that commonly observed VFA proportions in the rumen could be a result of thermodynamic equilibria controlling the diversion of carbon towards the different end products.

Calculations from VFA interconversion experiments also show that acetate synthesis may produce around 0.6 moles more ATP than propionate synthesis (not including secondary ATP generation from H_2 released). In addition, butyrate synthesis could produce a similar amount of ATP as acetate synthesis, but this has not been observed directly, suggesting that a great deal more heat is lost from butyrate synthesis or more ATP is produced by butyrate synthesis than previously thought.

The present analysis, based on published data from experiments where VFA interconversion was measured, has limitations. For example, none of these experiments measured gas concentrations,

so these were estimated. Also, all measurements were made in VFA extracellular pools, which, due to lack of ideal steady state, may have not completely equilibrated with intracellular pools. We need to conduct controlled experiments, specifically designed to measure all relevant concentrations, pressures and rates of fermentation in order to better quantify thermodynamic and kinetic parameters under different conditions. These additional data may make it possible to explain shifts in ruminal fermentation pathways.

It has been stated that "…we cannot manage, manipulate, or exploit systems that we do not understand" (Mackie and Bryant, 1994). Clearly, a great majority of the studies analyzed support the theory that the end products of ruminal fermentation are near equilibrium. And for many studies, having accepted this theory, we could have predicted the outcome. Finally, the use of thermodynamics to study the ruminal ecosystem may provide an example that is appropriate to other ecological or biological systems. Other systems where reactants are used rapidly and products are removed slowly include: silage, soil, manure digesters, and maybe some aspects of animal and plant metabolism. There are numerous proposals to manipulate gene expression to have an effect on enzymes. Understanding the thermodynamics of biological systems can contribute to design and optimize strategies for gene and enzyme manipulation. Understanding the thermodynamics of biological systems needs to be a high priority.

References

Alves de Oliveira, L., C. Jean-Blain, S. Komisarczuk-Bony, A. Durix & C. Durier (1997). Microbial thiamin metabolism in the rumen simulating fermenter (RUSITEC): the effect of acidogenic conditions, a high sulfur level and added thiamin. British Journal of Nutrition, 78, 599-613.

Armentano, L.E. & J.W. Young (1983). Production and metabolism of volatile fatty acids, glucose and CO_2 in steers and the effects of Monensin on volatile fatty acids kinetics. Journal of Nutrition, 113, 1265-1277.

Asanuma, N., M. Iwamoto & T. Hino (1999). Effect of the addition of fumarate on methane production by ruminal microorganisms in vitro. Journal of Dairy Science, 82, 780-787.

Barry, T.N., A. Thompson & D.G. Armstrong (1977). Rumen fermentation studies on two contrasting diets. 1. Some characteristics of the in vivo composition of the gas phase, oxidation/reduction state and volatile fatty acid proportions. J. Agric. Sci. Camb. 89: 183-195.

Bergman, E.N., R.S. Reid, M.G. Murray, J.M. Brockway & F.G. Whitelaw (1965). Interconversions and production of volatile fatty acids in the sheep rumen. Biochemical Journal, 97, 53-58.

Boone, D.R., R.L. Johnson & Y. Liu (1989). Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems and its implications in the measurement of K_m for H_2 or formate uptake. Applied and Environmental Microbiology, 55, 1735-1741.

Breznak, J.A. & M.D. Kane (1990). Microbial H_2/CO_2 acetogenesis in animal guts: nature and nutritional significance. FEMS Microbiology Reviews, 87, 309-314.

Bruce, L.A., G.E. Lobley & J.C. MacRae. 1987. Measurement of volatile fatty acid production in sheep given roughage. Research in Veterinary Science, 42, 47-52.

Callaway, T.R. & S.A. Martin (1996). Effects of organic acid and monensin treatment on in vitro mixed ruminal microorganism fermentation of cracked corn. Journal of Animal Science, 74, 1982-1989.

Carro, M.D. & M.J. Ranilla (2003a). Influence of different concentrations of disodium fumarate on methane production and fermentation of concentrate feeds by rumen micro-organisms *in vitro*. British Journal of Nutrition, 90, 617-623.

Carro, M.D. & M.J. Ranilla (2003b). Effect of the addition of malate on *in vitro* rumen fermentation of cereal grains. British Journal of Nutrition 89, 181-188.

Chang, R. (1981) Physical Chemistry with Applications to Biological Systems. 2nd ed. MacMillan Publishing Co., Inc., 659pp.

Clark, J.H. & C.L. Davis (1983). Future improvement of milk production: potential nutritional improvement. Journal of Animal Science, 57, 750-764.

Cord-Ruwisch, R., H.-J. Seitz & R. Conrad (1988). The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the electron acceptor. Archives for Microbiology, 149, 350-357.

Czerkawski, J.W. (1986). An Introduction to Rumen Studies. Pergamon Press, Oxford, UK, 236pp.

Demeyer, D. & H. Henderickx (1967). Competitive inhibition of "in vitro" methane production by mixed rumen bacteria. Archives Internationals de Physiologie et de Biochimie, 75, 157-159.

Esdale, W.J., G.A. Broderick & L.D. Satter (1969). Measurement of ruminal volatile fatty acid production from alfalfa hay or corn silage rations using a continuous infusion isotope dilution technique. Journal of Dairy Science, 51, 1823-1830.

Fogg, P.G.T. and W. Gerrard. (1985). Solubility of Gases in Liquids: A Critical Evaluation of Gas/Liquid Systems in Theory and Practice. John Wiley & Sons, Chichester, 332pp.

Fievez, V., F. Piattoni, L. Mbanzamihigo & D. Demeyer (1999). Reductive acetogenesis in the hindgut and attempts to its induction in the rumen –a review. Journal of Applied Animal Research, 16, 1-22.

Gray, F.V., G.B. Jones & A.F. Pilgrim (1960). The rate of production of volatile fatty acids in the rumen. Australian Journal of Agricultural Science, 11, 383-388.

Gray, F.V., A.F. Pilgrim, H.J. Rodda & R.A. Weller (1952). Fermentation in the rumen of sheep. IV. The nature and origin of the volatile fatty acids in the rumen of sheep. Journal of Experimental Biology, 29, 57-65.

Gray, F.V., R.A. Weller & G.B. Jones (1965). The rates of production of volatile fatty acids in the rumen. II. Measurement of production in an artificial rumen and application of the isotope dilution technique to the rumen of a sheep. Australian Journal of Agricultural Research, 16, 145-157.

Hackstein, J.H.P. (1997). Genetic and evolutionary aspects of methanogenesis. In A. Chesson, C.S. Stewart & H.J. Flint (eds.) Evolution of the Rumen Microbial Ecosystem, p. 5-8 . Proceedings of a Symposium jointly organized by The Rowett Research Institute and Inra, Centre de Clermont-Theix. Reproduction Nutrition Development (Suppl.).

Henderson, C. (1973). The effects of fatty acids on pure cultures of rumen bacteria. Journal of Agricultural. Science (Cambridge), 81, 107-112.

Hino, T. & N. Asanuma (2003). Suppression of ruminal methanogenesis by decreasing the substrates available to methanogenic bacteria. Nutrition Abstracts and Reviews Series B: Livestock and Feeding, 73, 1R-8R.

Holloway, P.E. & S.K. Baker (2002). Natural immune responses in sheep to methanogenic archaea. Reproduction Nutrition Development, 42 (Suppl. 1), S13.

Hungate, R.E., R.A. Mah & M. Simesen (1961). Rates of production of individual volatile fatty acids in the rumen of lactating cows. Applied Microbiology, 9, 554-561.

Iwamoto, M., N. Asanuma & T. Hino (1999). Effects of nitrate combined with fumarate on methanogensis, fermentation, and cellulose digestion by mixed ruminal microbes *in vitro*. Animal Science Journal, 70, 471-478.

Jalč, D. & Z. Čerešňáková (2001). Effect of plant oils and aspartate on rumen fermentation *in vitro*. Journal of Animal Physiology and Animal Nutrition, 85, 378-384.

Jalč, D, S. Kišidayová & F. Nerud (2002) Effect of plant oils and organic acids on rumen fermentation *in vitro*. Folia Microbiologica 47, 171, 177.

Joblin, K.N. (1999). Ruminal acetogens and their potential to lower ruminant methane emissions. Australian Journal of Agricultural Research, 50, 1307-1313.

Johnson, D.E., M. Branine, G.M. Ward, B. Carmean & D. Lodman (1991). The potential contribution of beef cattle methane to global warming: background information and perspectives. Colorado State University Beef Progress Report, 87-94.

Jou, D. and Llebot, J.E. (1990). Introduction to the Thermodynamics of Biological Processes. Prentice Hall, Englewood Cliffs.

Kohn, R.A. & R.C. Boston (2000). The role of thermodynamics in controlling rumen metabolism. In J.P. McNamara, J. France & D.E. Beever (eds.) Modelling Nutrient Utilization in Farm Animals. p 11-24. CAB International, Walingford, Oxon, UK, New York.

Kröger, A. & E. Winkler (1981). Phosphorylative fumarate reduction in *Vibrio succinogenes*: stoichimetry of ATP synthesis. Archives of Microbiology, 129, 100-104.

Kolver, E.S., P.W. Aspin, G.N. Jarvis, K.M. Elborough & J.R. Roche (2004). Digestion of pasture in response to fumarate in continuous culture. Journal of Animal Science 82 (Suppl. 1), 159.

Leng, R.A. & D.J. Brett (1966). Simultaneous measurement of the rates of production of acetic, propionic and butyric acids in the rumen of sheep on different diets and the correlation between production rates and concentrations of these acids in the rumen. British Journal of Nutrition, 20, 541-552.

Leng, R.A. & G.J. Leonard (1965). Measurement of the rates of production of acetic, propionic and butyric acids in the rumen of sheep. British Journal of Nutrition, 19, 469-484.

Le Van, T. D., J.A. Robinson, J. Ralph, R.C. Greening, W.J. Smolenski, J.A. Leedle & D.M. Schaefer (1998). Assessment of reductive acetogenesis with indigenous ruminal bacterium populations and *Acetitomaculum ruminis*. Applied and Environmental Microbiology, 64, 3429-3436.

Long, C. (1961). Biochemist's Handbook. E. & F.N. Spon, London, 1192pp.

López, S., F.M. McIntosh, R.J. Wallace & C.J. Newbold (1999a). Effect of adding acetogenic bacteria on methane production by mixed rumen microorganisms. Animal Feed Science and Technology, 78, 1-9.

López, S., C.J. Newbold, O. Bochi-Brum, A.R. Moss & R.J. Wallace (1999b). Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation *in vitro*. South African Journal of Animal Science, 29, 106-107.

López, S., C. Valdés, C.J. Newbold & R.J. Wallace (1999c). Influence of sodium fumarate on rumen fermentation *in vitro*. British Journal of Nutrition, 81, 59-64.

Mackie, R.I. & M.P. Bryant (1994). Acetogenesis in the rumen: synthropic relationships. In H.L. Drake (ed.) Acetogenesis, pp 331-364. Chapman & Hall, New York, London.

Maczulak, A.E., B.A. Dehority & D.L. Palmquist (1981). Effects of long-chain fatty acids on growth of rumen bacteria. Applied Environmental Microbiology, 42, 856-862.

Martin, S.A. (1998) Manipulation of ruminal fermentation with organic acids: a review. Journal of Animal Science 76, 3123-3132.

Martin, S.A. & M.N. Streeter (1995) Effect of malate on in vitro mixed ruminal microorganism fermentation. Journal of Animal Science, 73, 2141-2145.

Marwaha, S.R., M. Kaur, S. Singh, M.L. Punj & I.S. Bhatia (1972). *In vitro* studies on the effect of sodium sulphate and sodium tiosulphate on the production and interconversion of volatile fatty acids by rumen micro-organisms. Indian Journal of Animal Science, 42, 94-98.

McDonald, P., R.A. Edwards, J.F.D. Greenhalgh & C.A. Morgan (1995) Animal Nutrition. 5 th ed. Longman, Edinburgh Gate, Harlow, Essex, UK, 607pp.

Morvan, B., J. Doré, F. Rieu-Lesme, L. Foucat, G. Fonty & P. Gouet (1994). Establishment of hydrogen-utilizing bacteria in the rumen of the newborn lamb. FEMS Microbiology Letters, 117, 249-256.

Moss, A.R. (1993). Methane. Global Warming and Production by Animals. 1st ed. Chalcombe Publications, Kingston, Kent, UK, 105pp.

Müller, V. & G. Gottschalk (1994). The sodium ion cycle in acetogenic and methanogenic bacteria: generation and utilization of a primary electrochemical sodium ion gradient. In: H.L. Drake (ed.) Acetogenesis. P 125-156. Chapman & Hall, New York; London.

Nagaraja, T.G., C.J. Newbold, C.J. Van Nevel & D.I. Demeyer (1997). Manipulation of ruminal fermentation. In P.N. Hobson & C.S. Stewart (eds.) The Rumen Microbial Ecosystem. pp. 523-632. Blackie Academic & Professional, London.

Nakamura, Y., J. Yoshida, R. Nakamura & H. Horie (1975). Nitrate metabolism of microorganisms in the rumen of sheep fed high nitrate forages. Japanese Journal of Zootechnical Science, 47, 63-67.

Newbold, C.J., J.O. Ouda, S. López, N. Nelson, H. Omed, R.J. Wallace & A.R. Moss (2001) Propionate precursors as possible alternative electron acceptors to methane in ruminal fermentation. In J. Takahashi, B.A. Young & K. Umetsu (organizers) Greenhouse Gases and Animal Agriculture. pp. 272-275. Obihiro, Hokkaido, Japan.

Nollet, L., D. Demeyer & W. Verstraete (1997). Effect of 2-bromoethanesulfonic acid and *Peptostreptococcus productus* ATCC 35244 addition on stimulation of reductive acetogenesis in the ruminal ecosystem by selective inhibition of methanogenesis. Applied and Environmental Microbiology, 63, 194-200.

Oba, M. & M.S. Allen (2003). Extent of hypophagia caused by propionate infusion is related to plasma glucose concentration in lactating dairy cows. Journal of Nutrition, 133, 1105-1112.

Ørskov, E.R. & M. Ryle (1990). Energy Nutrition in Ruminants. 1st ed. Elsevier Science Publishers Ltd., Essex, UK, 149pp.

Prins, R.A., C.J. Van Nevel & D.I. Demeyer (1972). Pure culture studies of inhibitors for methanogenic bacteria. Antonie Van Leeuwenhoek, 38, 281-287.

Rekharsky, M.V., Galchenko,G.L., Egorov, A.M. & I.V. Berenzin. (1986). Thermodynamics of enzymatic reactions. In: Hinz, H.-J. (ed.) Thermodynamic Data for Biochemistry and Biotechnology. Springer-Verlag, Berlin, pp. 431-444.

Reddy, C.A. & H.D. Peck (1978). Electron transport phosphorylation coupled to fumarate reduction by H_2- and Mg^{2+} -dependent adenosine triphosphate activity in extracts of the rumen anaerobe *Vibrio succinogenes*. Journal of Bacteriology, 134, 982-991.

Russell, J.B. (1991) Resistance of *Streptococcus bovis* to acetic acid at low pH: relationship between intracellular pH and anion accumulation. Applied and Environmental Microbiology, 57, 255-259.

Russell, J.B. (2002) Rumen Microbiology and Its Role in Ruminant Nutrition, 1st ed. Ithaca, NY.

Russell, J.B. & R.J. Wallace (1997). Energy-yielding and energy-consuming reactions. In: P.N. Hobson & C.S. Stewart (eds.) The Rumen Microbial Ecosystem. pp. 246-282. Blackie Academic and Professional, London.

Samuelov, N.S., R. Datta, M.K. Jain & J.G. Zeikus (1999). Whey fermentation by *Anaerobiospirillum succinoproducens* for production of a succinate-based animal feed additive. Applied and Environmental Microbiology, 65, 2260-2263.

Schulman, M.D. and D. Valentino (1976). Factors influencing rumen fermentation: effect of hydrogen on formation of propionate. Journal of Dairy Science, 59, 1444-1451.

Segel, I.H. (1976). Biochemical Calculations, 2nd edn. John Wiley & Sons, New York.

Seal, C.J. & D.S. Parker (1994). Effect of intraruminal propionic acid infusion on metabolism of a mesenteric- and portal-drained viscera in growing steers fed a forage diet: I. Volatile fatty acids, glucose, and lactate. Journal of Animal Science, 72, 1325-1334.

Sharp, W.M., R.R. Johnson & F.N. Owens (1982). Ruminal VFA production with steers fed whole or ground corn grain. Journal of Animal Science, 55, 1505-1514.

Sheppard, A.J., R.M. Forbes & B.C. Johnson (1959). Rate of acetic acid production in the rumen determined by isotope dilution. Proceedings of the Society for Experimental Biology and Medicine, 101, 715-717.

Takahashi, J. (2001). Nutritional manipulation of methane emission from ruminants. In Greenhouse Gases and Animal Agriculture pp. 62-71. Obihiro, Hokkaido, Japan.

Thauer, R.K., K. Jungermann & K. Decker (1977). Energy conservation in chemotrophic anaerobic bacteria. Bacteriological Reviews, 41, 100-180.

Ungerfeld, E.M., S.R. Rust & R. Burnett (2003). Use of novel alternative electron sinks to inhibit ruminal methanogenesis. Reproduction Nutrition Development, 43, 189-202.

Van Campen, D.R. & G. Matrone (1960). Investigation of precursors of ruminal fatty acids of sheep fed purified diets. Journal of Nutrition, 72, 277-282.

Voet, D. & J.G. Voet (1995). Biochemistry. 2nd ed. John Wiley & Sons, Inc., New York, 1361pp.

Wallace, R.J., R. Onodera & M.A. Cotta (1997). Metabolism of nitrogen-containing compounds. In: P.N. Hobson & C.S. Stewart (eds.) The Rumen Microbial Ecosystem pp. 283-328. Blackie Academic & Professional, London.

Weller, R.A., F.V. Gray, A.F. Pilgrim & G.B. Jones (1967). The rates of production of volatile fatty acids in the rumen. IV. Individual and total volatile fatty acids. Australian Journal of Agricultural Research, 18, 107-118.

Wolin, M.J., T.L. Miller & C.S. Stewart (1997). Microbe-microbe interactions. In: P.N. Hobson & C.S. Stewart (eds.) The Rumen Microbial Ecosystem pp. 467-491. Blackie Academic & Professional, London.

Digestion and passage of fibre in ruminants

P. Huhtanen[1], S. Ahvenjärvi[1], M.R. Weisbjerg[2] and P. Nørgaard[3]
[1]MTT-Agrifood Research Finland, Animal Production Research, FIN-31600 Jokioinen, Finland
[2]Danish Institute of Agricultural Sciences, Department of Animal Health, Welfare and Nutrition, Research Centre Foulum, DK-8830 Tjele, Denmark
[3]The Royal Veterinary- and Agricultural University, Department of Animal and Veterinary Basic Sciences, DK-1870 Frederiksberg, Denmark

Abstract

The objective of this chapter is to discuss the methods used for the estimation of digestion and passage kinetic parameters, intrinsic and extrinsic characteristics influencing the kinetic parameters and the reliability of kinetic parameters for predicting *in vivo* digestibility using dynamic rumen models. We focus mainly on digestion and passage of cell wall carbohydrates, since most of the variation in organic matter digestibility of ruminant diets can be attributed to concentration and digestibility of cell wall carbohydrates.

The extent and rate of NDF digestion are generally determined by *in situ* or *in vitro* methods, but estimates have seldom been validated using *in vivo* data. A method for estimating digestion rate from *in vivo* digestibility of potentially digestible NDF and assumed rumen residence time is suggested. Future work is required to estimate the intrinsic characteristics limiting rate and extent of cell wall digestion, and the quantitative effects of some extrinsic factors such as intake and diet composition.

Extensive data suggest that most of the compartmental retention time in cattle is pre-duodenal and that the reticulo-rumen is a system with selective retention of feed particles. However, models with selective retention have seldom been used to calculate NDF digestibility from kinetic parameters. This fundamental flaw in the model structure leads to serious underestimations of NDF digestibility unless unrealistically high digestion rates and/or low passage rates are used to correctly predict *in vivo* digestibility. To progress in understanding of NDF digestion, it is vital to develop useful mechanistic models for the prediction of digestibility and intake.

Keywords: cell wall carbohydrate, digestion kinetics, passage kinetics, intrinsic factor, extrinsic factor, modelling

Introduction

Animal performance depends on the intake of digestible and metabolisable nutrients. Although a large proportion (60-90%) of the variation in digestible energy (DE) intake is related to differences in intake (see Mertens, 1994), differences in diet digestibility also have a significant effect on nutrient supply. In addition to the direct effects on DE intake, digestibility also influences nutrient supply indirectly due to the close association between digestibility and intake in ruminants fed forage-based diets. In dairy cows fed grass silage based diets improvements in silage digestibility

were closely related to increased dry matter (DM) intake and animal performance (Rinne, 2000).

The concept of an ideal nutritional entity was initially proposed by H.L. Lucas. According to the Lucas principle, the true digestibility of a nutritional entity is determined as the slope of the regression between the amounts of nutrient digested (e.g. crude protein, ether extract, and cell solubles) against the intake of a given nutrient (Van Soest, 1994). The negative intercept of this regression represents faecal metabolic output. The true digestibility of cell solubles defined as DM - neutral detergent fibre (NDF) was found to be 0.98 and not significantly different from 1.00 (Van Soest, 1994). When cell solubles are defined as organic matter (OM) minus NDF, the true digestibility of cell solubles in grass silages from primary growths was complete, and the standard errors of both the slope and intercept were small (Nousiainen *et al*., 2003a). Applying the Lucas principle to a larger data set with a wider range of diet composition from digestibility studies in sheep demonstrated a small variation in the true digestibility of cell solubles (Weisbjerg *et al*., 2004a). Cell wall characteristics (dietary NDF concentration and digestibility) explained variation in OM digestibility with prediction errors of less than 10 g kg^{-1} within a study across three data sets (Nousiainen *et al*., 2004; unpublished data). This indicates that OM digestibility, which is the key factor in determining the DE concentration of a feed, is primarily constrained by the cell wall characteristics. The primary importance of cell wall characteristics in assessing OM digestibility of the diet does not imply that other dietary components are not important. For example, starch digestibility is influenced by grain source and physical processing (Firkins *et al*., 2001).

The availability and digestion passage kinetics of different carbohydrates are summarised in Table 1. Both soluble carbohydrates and soluble cell wall carbohydrates like β-glucans and pectins are readily degraded in the rumen, and only minor parts will escape for post-ruminal digestion except for some slowly degradable starch from e.g. maize. Most soluble carbohydrates are digestible in both the small intestine and the hind-gut, if they escape rumen degradation. Insoluble cell wall carbohydrates are generally slowly degraded in the rumen, and therefore the extent of rumen digestion is highly dependent on residence time of the fibre in the rumen. As digestion of both soluble and insoluble fibre is solely dependent on microbial fermentation, no digestion will take place in the small intestine; however fibre that escapes rumen digestion may be degraded in the hind-gut.

Digestion of dietary entities is a time-dependent process. The proportion of a nutrient that becomes available for absorption is determined by the rate of digestion relative to the rate of passage. For cell walls the rate of digestion in relation to passage is very slow compared with cell solubles, which explains the larger variability in cell wall digestibility. Digestion and passage in ruminants can be mechanistically described by compartmental models of varying complexity. Illius and Allen (1994) made a detailed comparison of the structure and assumptions of the models, which differed principally in the fractioning of feed and in the description of digestion and passage kinetics. Forage digestibility predicted by these models is generally within 15% of observed values (Illius and Allen, 1994), with R^2 values between observed and predicted in the range of 0.5 to 0.7. For practical feed evaluation, these models are simply not accurate enough. The bias in model predictions that frequently occur are more likely to result from a poor estimation of digestion

Table 1. Availability and digestion rate of different carbohydrate fractions.

	Rumen	Small intestine	Hind gut	Digestion rate
Cell solubles				
Sugars	High	(High)[1,2]	(High)[1]	Very fast
Starch	High (variable)	Variable	Variable	Fast
Soluble fibre				
Pectins, β-glucans	High	0	(High)[1)]	Fast?
Insoluble fibre				
NDF	Variable	0	Variable	Slow

[1] Only very little will reach post-duodenal digestive tract
[2] Some exceptions like sucrose

and passage kinetic parameters than from fundamental problems in model structure (Illius and Gordon, 1991). Much research has been conducted during the last decades to estimate digestion and passage kinetic parameters. However, most of the studies on digestion kinetics of cell walls (NDF) have compared parameter values between feeds, often estimating ruminal digestibility using simple dynamic rumen models. Curve fitting and the validity of kinetic methodology has also been extensively studied. However, the performance of models using digestion kinetic parameters has seldom been validated. In studies of passage kinetics the main focus has been in comparing markers, fitting marker concentration data to various compartmental models and particle size analysis, with less emphasis on validation of the data using dynamic rumen models. The lack of rigorous testing and evaluation of digestion and passage kinetic parameters in dynamic rumen models has probably prevented progress in developing useful models for accurate and reliable prediction of diet digestibility in ruminant animals. Experimental work and modelling should be carried out in harmony to reach satisfactory model performance.

The objectives of this chapter are to discuss the methods used for the estimation of digestion and passage kinetic parameters (1), intrinsic and extrinsic characteristics influencing the kinetic parameters (2) and the reliability of kinetic parameters for predicting *in vivo* digestibility using dynamic rumen models (3). Because most of the variation in OM digestibility can be explained by the characteristics of the cell wall fraction, the main focus will be placed on NDF digestion.

Site of digestion

In cattle the major proportion of cell wall digestion occurs in the forestomach. Paloheimo and Mäkelä (1959) examined the residence time in different digestion compartments in a slaughter study with dairy cows (n = 21) that consumed between 5-20 g DM per kg LW. Based on lignin pool sizes the mean fractional residence time of particulate matter was 0.76 in the reticulo-rumen, 0.10 in the omasum, 0.05 in the abomasum and small intestine and 0.10 in the caecum and colon. The proportion of NDF digestion in the forestomach (i.e. reticulo-rumen and omasum) is higher than indicated by the mean residence time because the potential digestibility [DNDF(digestible NDF)/NDF] of particulate matter decreases with time as digesta passes through successive compartments. Digestibility in the hindgut (i.e. caecum and colon) is therefore dependent on

the extent of forestomach NDF digestion. Sub-optimal rumen conditions decrease the digestion rate relative to that under optimal conditions, lower NDF digestibility in the forestomach and increase the amount of digestible NDF (DNDF) entering the hindgut. However, the efficiency of the hindgut microbial population to digest fibre seems to be lower than that of rumen microbes under normal rumen conditions. Michalet-Doreau *et al*. (2002) reported markedly lower cellulolytic activities in the caecum than in the rumen, suggesting that the rate of fibre digestion in the caecum and colon was lower than that in the rumen.

Flow studies have consistently indicated that the forestomach is the major site of NDF digestion in cattle (Tamminga, 1993). Based on three studies in duodenally cannulated cattle, the proportion of NDF digestion occurring in the forestomachs was on average 0.97 (Table 2). In seven other studies with lactating dairy cattle, NDF digestibility in the reticulo-rumen was determined based on the flow of NDF entering the omasal canal. The mean proportion of total tract NDF digestion in the reticulo-rumen was 0.93 in those studies. Because the role of the omasum appears to be larger than that of the hindgut in NDF digestion in cattle (Paloheimo and Mäkelä, 1959; Ahvenjärvi *et al*., 2001) these results indicate that typically less than 0.05 of total NDF digestion takes place in the hindgut.

In order to illustrate the effects of suboptimal rumen conditions on NDF digestion occurring at different sites and the potential of hindgut digestion to compensate for decreased forestomach digestibility a simulation study on DNDF digestibility in the forestomach, hindgut and total tract was conducted. The model consisted of two rumen pools, a non-escapable pool with a second order gamma distribution of residence times, an escapable pool with a first order passage rate,

Table 2. Fractional proportion of NDF digestion in the intestines determined based on duodenal NDF flow and NDF digestion in the omasum and intestines determined based on omasal canal NDF flow.

Reference	Animal species	Diets, n	DM intake, kg^{-1} LW	Digesta sampling site	Fractional proportion of NDF digested proximal to the sampling site
Rinne *et al*., 1997	Cattle	4	16	Duodenum	1.01
Huhtanen and Jaakkola, 1993	Cattle	6	18	Duodenum	0.93
Khalili and Huhtanen, 1991	Cattle	4	17	Duodenum	0.98
Stensig and Robinson, 1997	Cow	4	-	Duodenum	1.01
Volden, 1999	Cow	6	16 and 32	Duodenum	0.81 and 0.92
Lund, 2002	Cow	14	22	Duodenum	1.00
Ahvenjärvi *et al*., 1999	Cow	4	26	Omasal canal	0.90
Korhonen *et al*., 2002	Cow	4	30	Omasal canal	0.86
Ahvenjärvi *et al*., unpublished	Cow	4	35	Omasal canal	0.89
Ahvenjärvi *et al*., unpublished	Cow	4	31	Omasal canal	0.99
Ahvenjärvi *et al*., unpublished	Cow	4	32	Omasal canal	1.00
Kuoppala *et al*., unpublished	Cow	4	31	Omasal canal	0.92
Shingfield *et al*., unpublished	Cow	4	30	Omasal canal	0.96

and a pool for the omasum (mixing pool), abomasum and small intestine (tubular flow), caecum and proximal colon (mixing pool) and distal colon (tubular flow). Further, the model assumed a total mean residence time of 46 h (Huhtanen and Hristov, 2001; Ahvenjärvi *et al*., 2004), which was allocated between compartments based on the distribution between compartments reported by Paloheimo and Mäkelä (1959). Sub-optimal rumen conditions were assumed to decrease the rate of DNDF digestion from 0.075 to 0.030 h^{-1}, while the rate of DNDF digestion in the hindgut was assumed to be optimal (0.075), irrespective of rumen conditions. The results indicate that within the given range of the rate of digestion, DNDF digestibility in the forestomach decreased from 0.81 to 0.56 and that in the total tract from 0.85 to 0.66 (Figure 1).

The DNDF digestibility in the hindgut increased from 0.04 to 0.10 while the proportion of DNDF digestion in the hindgut as a proportion of total digestion increased from 0.05 to 0.16. These simulated results clearly suggest that due to limited residence time, the capacity of the hindgut to digest fibre is limited and can only partly compensate for lowered digestion in the forestomach. Consistent with flow measurements and modelling approaches, Huhtanen and Vanhatalo (1997) found using a combined rumen *in situ* incubation and mobile bag technique that the contribution of the hind-gut to the total NDF digestion was small. In the case that the hind-gut fermentation requires microbial colonisation (lag time), the extent of the cell wall digestion in the hindgut of ruminants would be further limited.

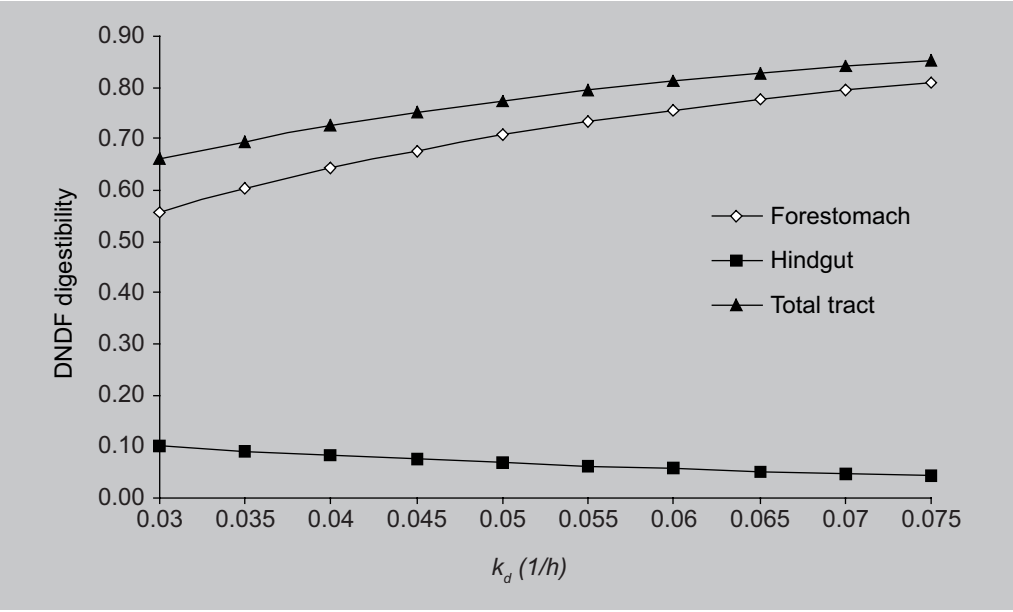

Figure 1. Simulated effects of digestion rate (k_d) in the forestomach on DNDF digestibility in the forestomach (reticulo-rumen and the omasum), hindgut (caecum and colon) and the total tract.

Digestion kinetics

The digestive system of ruminants is well adapted to the utilization of cell walls by microbial fermentation and the specialised ruminant stomach is comprised of four compartments (rumen, reticulum, omasum and abomasum). Fermentation of cell walls occurs in the first three compartments in a complex ecosystem that is influenced by interactions between feeds, microbial populations and the host animal. The rumen and reticulum form a large fermentation chamber (up to 20% of body weight) containing an active and diverse microbial population. Physical breakdown of large particles to small particles by mastication during ingestion and rumination is an important part of digestion process in ruminants. An optimal pH for microbial fermentation of cell wall carbohydrates is maintained by continuous salivary flow and absorption of volatile fatty acids (VFA) produced during fermentation. The role of the omasum, which is more developed in cattle than in sheep, is not completely understood. It appears to be related to the absorption and selective retention of feed particles in the rumen. In cattle, the omasum may have a greater role in NDF digestion than the intestines (Ahvenjärvi *et al*., 2001). Microbial fermentation of carbohydrates is completed in the large intestine which behaves like a hybrid mixing-plug flow reactor.

Since the proposal of the NDF analysis as a measure of insoluble fibre some 40 years ago (Van Soest, 1963), this analysis has gained popularity and is now generally accepted as the most appropriate analysis for the determination of cell wall content of ruminant feeds. Although the NDF fraction does not include certain cell wall materials such as pectins and β-glucans, measurement of this entity does separate the completely digestible fraction from insoluble and partially digestible nutrients. Pectins and β-glucans are rapidly fermented and almost completely digested in the ruminant digestive tract (Van Soest, 1994), whereas the digestibility of other cell wall carbohydrates is highly variable due to differential lignification.

Parameter estimates of intrinsic rate and extent of digestion

Accurate and precise predictions of the intrinsic digestion kinetic parameters are critical to the accurate prediction of NDF digestibility. However, complicated symbiotic interactions between rumen microbes, the diet and host animal are essential for the utilisation of nutrients from cell walls. In order to be useful in dynamic rumen models, the kinetic parameters should only be limited by the attributes of substrates, i.e. intrinsic characteristics of cell walls. Physical and chemical attributes of the digestion environment should not be limiting factors in the determination of the potential rate and extent of NDF digestion

Several reviews of digestion kinetics of cell wall carbohydrates (Mertens, 1993a, 1993b; Ellis *et al*., 1994, 1999) have addressed the problems associated with the estimation of kinetic parameters. The following discussion will focus primarily on the problems related to the determination of kinetic parameters and the importance of both accuracy and precision of these measurements. Errors in kinetic variables used for data validation limit improvement in mechanistic models as much as for empirical models. The importance of the rate and extent of NDF digestion on OM and NDF digestibility, rumen NDF pool and microbial N flow can be demonstrated by the Nordic model of dairy cow metabolism "Karoline" (Danfær *et al*., 2005a,b). Simulations were made for a 550

kg dairy cow consuming 15 kg d^{-1} of grass silage DM using a range of indigestible NDF (INDF) concentrations and rates of digestible NDF (DNDF) digestion (Table 3). Simulation results clearly demonstrate profound effects of these parameters on OM digestibility and consequently on the supply of energy and microbial protein.

The earliest attempts to describe the kinetics of digestion have been reviewed by Mertens (1993a, 1993b). The term "rate of digestion" appeared in the 1950s, but the assessments were mainly based on the visual interpretation of digestion curves. The major breakthrough was made by Waldo (1970), who suggested that digestion curves are a combination of indigestible and digestible material. He also suggested that if the indigestible residue was subtracted, digestion of potentially digestible cell walls might follow first-order kinetics. The hypothesis that some material is indigestible was based on earlier work of Wilkins (1969), who observed that some cellulose remained undigested after extended periods of fermentation. Smith *et al*. (1972) used 72 h *in vitro* fermentations to determine indigestible NDF content (INDF). The potentially digestible NDF residue at earlier fermentation times was estimated by subtracting INDF from total NDF residue. The regression between the natural logarithm of DNDF against time was linear supporting the hypothesis that DNDF follows the first-order digestion kinetics. Indigestible NDF is an ideal nutritional entity according to the Lucas principle, because by definition it is digested at a predictable rate of zero. According to Ellis *et al*. (1999) determination of INDF should be included in all basic feedstuff analysis because (1) it has a predictable digestibility; (2) it can be used for the estimation of DNDF as NDF-INDF and (3) it has an important role in contributing to rumen digesta load.

The in situ method

The *in situ* method is the most common method used to estimate NDF digestion kinetic parameters. Several excellent reviews (Nocek, 1988; Mertens, 1993b; Ellis *et al*., 1994; Stern *et al*., 1997; Nozière and Michalet-Doreau, 2000) have been published that provide a detailed insight into the sources of variation and methodological aspects of the procedure. Regardless of the method used to generate kinetic data, the system should measure the intrinsic rate of digestion,

Table 3. The effects of INDF[1] concentration and fractional rate of DNDF digestion (k_d) on total digestibility, rumen NDF pool and microbial N flow simulated by the Nordic dairy cow model (Danfær et al.*, 2005a).*

	INDF (g kg^{-1} DM)			k_d (h^{-1})			
	60	**100**	**140**	**0.04**	**0.05**	**0.06**	**0.07**
Digestibility							
OM	0.733	0.700	0.667	0.663	0.692	0.712	0.727
NDF	0.727	0.673	0.620	0.615	0.663	0.697	0.720
DNDF	0.808	0.808	0.808	0.739	0.795	0.836	0.865
NDF pool (kg)	6.76	7.47	8.18	7.91	7.20	6.65	6.25
Microbial N flow (g d^{-1})	227	213	197	203	216	226	233

[1]INDF concentrations (60, 100 and 140 g kg^{-1} DM) correspond to potential NDF digestibility of 0.900, 0.833 and 0.767, respectively.

which requires that the system itself does not limit digestion. The number of data points collected should be sufficient, particularly at the beginning and end of fermentation, to establish the initial solubilisation/lag and potential extent of fermentation (Mertens, 1993b). However, most of the published data on NDF digestion kinetics have been determined using less sampling times than what Mertens (1993b) suggested being optimal.

Several kinetic models to describe NDF digestion kinetics have been proposed (Mertens, 1993a, 1993b; Ellis *et al*., 1994). The models differ with respect to the assumptions of a partition between potentially digestible and indigestible fibre (1), the number of compartments having a homogenous rate of digestion (2), a discrete lag time vs. compartmental lag time (3), time-independent vs. time-dependent distribution of digestion rates in the compartment (4) and first-order vs. second-order digestion kinetics (5). Although the assessment of INDF is critical for the accurate estimation of kinetic parameters, too often the kinetic parameters are calculated without accounting for the indigestible residue or by using a value determined over too short a fermentation time (Mertens, 1993a) when using linear regression on natural logarithm transformed DNDF residue. Using a model with heterogeneous rates provided superior or at least as good a fit compared with first-order models (Ellis *et al*., 1994). The use of this model may be justified by the heterogeneous nature of chemical entities and their physical distribution in diverse plant tissues (Van Soest *et al*., 2000). However, Van Milgen *et al*. (1993) recommended the use of first-order models because they provide rates that are easily interpreted, in contrast to the parameters generated by models using heterogeneous rates. In addition, using heterogeneous rate parameters in dynamic mechanistic rumen models is more difficult than using a first-order rate parameter. These problems may be solved by using the mean rate for the heterogeneous rates models (Ellis *et al*., 1994), but in this case the possible advantages of a better model fit of the data are lost in the prediction of digestibility.

A plot of NDF residues against fermentation time often exhibits a lag period before the onset of fermentation (Mertens, 1993a). Lag is assumed to represent processes such as hydration of feeds, the time for microbial colonization and occurrence of analytically detectable digestion. The biological mechanisms underlying the lag phenomena are discussed in more detail by Allen and Mertens (1988) and Firkins *et al*. (1998). Mertens (1977) modified the first-order digestion model by including a discrete lag time. However, in biological systems it is unlikely that first-order digestion would start instantaneously after the lag period. Allen and Mertens (1988) proposed a two-compartmental sequential model (lag compartment and digestion compartment) to describe the process involving attachment of microbes to the cell walls followed by microbial digestion of cell walls. Van Milgen *et al*. (1991) proposed mathematical models that can be used to estimate the parameters for the sequential two-compartment model. This model affords a method for describing a less abrupt initiation of digestion. However, more work is needed on the biological accuracy of lag parameters and their importance to the accuracy and precision of NDF digestibility predictions by dynamic mechanistic rumen models. Allen and Mertens (1988) demonstrated by mathematical analysis, that if the lag phenomenon affect both digestion and passage, then the lag term has no influence on digestibility. Digestibility is independent of lag because wetting of particle is a prerequisite for both digestion and passage. However, a lag time on both digestion and passage will severely increase rumen load and alter the prediction of feed intake.

Two main methods are used for fitting data to the first-order kinetic models: linear regression on logarithmic transformations of undigested residues (ln-linear) and nonlinear estimation of parameters. Nonlinear models estimate parameter values simultaneously and assume an equal error at each fermentation time, whereas the ln-linear models assume that error is proportional to the size of residue at each time point. Neither of these approaches seems reasonable, because random errors are typically the largest for medium (8-48 h) incubation times. In the ln-linear approach indigestible NDF must be determined experimentally using the data from the last incubation time, and therefore any error in the estimation potential digestibility can bias the values for other parameters. For further details of the calculations of model parameter values the reader is referred to the reviews of Mertens (1993a, 1993b) and Ellis *et al.* (1994). Numerous models describing digestion kinetics have been evaluated by comparing the fit of the data, whereas robust testing of the kinetic models by comparing model predictions of digestibility with reference *in vivo* measurements is extremely limited.

Digestion of NDF continues even after long incubation periods *in situ* (Robinson *et al.*, 1986) suggesting that extended incubations are necessary in order to estimate INDF. Prolonged incubations also present other problems such as mineral precipitation occluding bag pores, escape of small particles from the bag or influx of material into the bag. These problems can partly be avoided by determining INDF on an ash-free basis and using bags of small pore size. A close empirical relationship between silage INDF content and OM digestibility (Nousiainen *et al.*, 2003b) indicates that INDF is a useful entity for the prediction of the nutritive value of forages. Indigestible NDF was determined by 12 d ruminal incubations in nylon bags of small pore size (6 or 17 μm). The relationship between INDF and OM digestibility was uniform for the primary growth and regrowth silages, whereas the relationship between OM pepsin-cellulase solubility and OM digestibility were different for the two types of silages. Near infrared reflectance spectroscopy (NIRS) can potentially be used for a rapid and accurate estimation of INDF content from forage samples (Nousiainen *et al.*, 2004). Ideally both the rate and extent of NDF digestion should be estimated simultaneously.

In vitro methods

Digestion kinetics can be evaluated *in vitro* from the disappearance of NDF or by measuring the volume of gas produced during the fermentation. When the methods are used to determine the intrinsic characteristics of feeds, it is important that the system does not impose constraints on digestion. Essential nutrients (e.g. ammonia, amino acids, and trace elements), pH, redox potential, anaerobicity and microbial numbers should not be limiting when measuring intrinsic characteristics of cell walls (Grant and Mertens, 1992). Variation in the activity of inoculum has been reported to affect the rate of NDF digestion (Cherney *et al.*, 1993). Variation between animals, species of the donor animal, feeding management, time of inoculum collection relative to feeding and the diet fed to the donor animal all affect *in vitro* digestibility (Weiss, 1994). These are animal factors which could also influence digestion kinetic parameters, of which the effect of diet is probably the most important. *In vitro* methodology has been extensively reviewed elsewhere (Mertens, 1993a; Weiss, 1994; Firkins *et al.*, 1998).

Automated methods to measure gas production system have some advantages relative to other methods. Frequent measurements can be made by the use of electronic pressure sensors and datalogging equipment. Automated data collection from the same fermentation vessel allows the collection of a sufficient number of observations for accurate parameter estimation. The second advantage is that digestion rates of different feed fractions can be estimated by fractionation of the feed before incubation. The NDF fraction is relatively easy to deal with because NDF can be chemically isolated and digestion kinetics can be measured by the gas production system (Schofield and Pell, 1995). If the digestion curve of NDF is subtracted from the equivalent amount of intact feed, a gas production curve for neutral detergent (ND) solubles is obtained and the kinetic parameters for ND solubles can be estimated from the latter curve. Estimation of digestion kinetics for ND solubles by the *in situ* method is not possible, because most of ND solubles escape the bag either by solubilisation or by efflux as small particles. Attempts have also been made to relate the pools estimated by a three-pool model to chemical fractions of a feed (Cone *et al*., 1997). Although some similarities were observed, the relationship was not consistent. The pools estimated by the multi-pool models should therefore be viewed as purely mathematical constructs that may or may not correspond to chemical entities (Schofield, 2000).

In their review, Firkins *et al*. (1998) referred to several problems of the gas production system including a correction for changes in fermentation stoichiometry (VFA ratio) over time, evolution of gas from the buffer, errors caused by small sample sizes, an inability of the system to distinguish between different substrates, the contribution of ammonia to the gas pool and problems related to the blank correction. Many of these problems can, however, be reduced by chemically isolating NDF and measuring its digestion behaviour *in vitro*. Comparative studies on NDF digestion in the whole forages and in isolated NDF suggest that both the extent and rate of NDF digestion are similar (Doane *et al*., 1997). The systems measuring digestion kinetics from gas production profiles have many common sources of errors with systems based on substrate disappearance, and it is equally important that digestion rate is not limited by the system. Technical details of automated gas production systems have been described in detail (Pell and Schofield, 1993; Theodorou *et al*., 1994; Cone *et al*., 1996).

Rumen evacuation technique

In the steady state situation the flux of an entity in or out of the rumen is related to compartmental mass. Fractional rates of intake, passage and digestion for the entities can be estimated by dividing these flows by rumen pool size (Robinson *et al*., 1987) using the following equations:

$$k_i \text{ (Rate of intake; } h^{-1}) = \text{Intake (kg } h^{-1}) \text{ / Rumen pool (kg)} \quad (1)$$

$$k_p \text{ (Rate of passage; } h^{-1}) = \text{Flow (kg } h^{-1}) \text{ / Rumen pool (kg)} \quad (2)$$

$$k_d \text{ (Rate of digestion; } h^{-1}) = k_i - k_p \quad (3)$$

For a meaningful interpretation, k_d must be estimated only for digestible NDF. Estimating k_d for total NDF, although reported for some rumen evacuation studies, is meaningless and of little value. On a biological basis, estimating k_d by rumen evacuation (flux method) for total NDF is

incorrect, because digestion rate is determined for a fraction that also contains INDF. Kinetically it is not correct, because the pools do not have homogenous kinetic characteristics. Different proportions of INDF and DNDF are present in the flux and rumen pool.

Theoretically, the rumen evacuation technique should be an ideal method for estimating digestion rate, but it does have some disadvantages. It is time-consuming, expensive and laborious precluding its use for routine analysis. An accurate estimation of rumen pool size requires steady-state conditions which are difficult to achieve even in *ad libitum* fed animals. This problem can be reduced, although not completely eliminated, by frequent rumen evacuations and careful selection of rumen evacuation times to represent the mean rumen pool size. Estimation of k_d and k_p for DNDF requires accurate duodenal DNDF flow measurements. This may not be a major problem since the contribution of post-ruminal compartments to total NDF digestion is small (see Table 2), especially when conditions in the rumen are not a limiting factor for digestion. Under these circumstances predicting duodenal DNDF flow from faecal output as suggested by Robinson *et al.* (1987) may not markedly increase the error of k_d estimates. Probably the single largest disadvantage of the technique is that only digestion parameters for the total diet rather than for individual feeds can be estimated. Rumen evacuation technique ignores omasal cell wall pools, which has some influence on estimated kinetic parameters. Assuming that INDF and DNDF pool sizes in the omasum represent proportionally 0.20 and 0.13 of that in the rumen, respectively (Ahvenjärvi *et al.*, unpublished data), passage rate would be overestimated by a factor of 1.25 for INDF, and by 1.15 for DNDF digestion and passage rates.

In vivo digestibility method

Digestibility coefficients measured in sheep fed at a maintenance level of feeding is still the basis of most feed evaluation systems. Because digestibility of DNDF is a function of digestion and passage rates, it might be possible to estimate digestion rate of DNDF if the values for the digestibility of DNDF and compartmental residence time were available. In digestion trials digestibility of DNDF can be calculated when INDF content of the feeds is determined. The DNDF digestibility can be calculated from the kinetic parameters using the two compartment model that incorporates selective retention of feed particles in the rumen (Allen and Mertens, 1988) as follows:

$$\text{DNDF digestibility } (D) = (k_d /(k_d + k_r)\ [1 + k_r /(k_{d+}\ k_p\ )] \quad (4)$$

where k_d, k_r and k_p are the rates of digestion, release from the non-escapable to the escapable compartment and passage to the lower tract. The rate of digestion can be solved from equation 4:

$$k_d = [-(k_p + k_r) + [(k_p + k_r)^2 + 4Dk_r k_p/(1 - D)]^{0.5}]/2 \quad (5)$$

To estimate the rate of digestion indirectly by this method, an estimate of total mean residence time in the fermentation compartments and the distribution of the residence time between the two compartments are required. A data set of 52 primary growth and regrowth grass silages harvested at different stages of maturity (Nousiainen *et al.*, 2003a; 2003b) was used to calculate digestion rate assuming a compartmental mean residence time (CMRT) of 50 h for sheep fed at

maintenance and a value of 0.30 for the proportion of total CMRT in the first compartment. The mean rate of DNDF digestion was 0.075 h^{-1} (s.d. 0.0163, range 0.050 - 0.117). The disadvantages of this approach are that it requires reliable estimates of the passage kinetic parameters and that the kinetic data are estimated retrospectively from end-point determinations.

However, two questions arise; (1) are the differences between individual feed passage kinetics important enough to be taken into account and (2) how accurately can they be determined using current methods compared with simplifying using a constant value or deriving empirical relationships between intake and residence time. Estimates of k_d values are not markedly influenced by small variations in CMRT. For example, an increase in CMRT from 50 to 55 h would decrease the mean k_d value from 0.060 to 0.055 h^{-1}. Also the distribution of CMRT between the two compartments has a relatively small influence on calculated k_d value when the proportion of total CMRT is within a range of 0.20 - 0.40 (0.058 - 0.065 h^{-1}).

In conventional feed evaluation the digestibility of OM in sheep at maintenance is routinely estimated using laboratory methods. Using the Lucas principle does allow the estimation of ND solubles digestibility which combined with OM digestibility enables NDF digestibility to be calculated (Weisbjerg *et al*., 2004b). If this approach is combined with INDF determination, estimates of k_d could be obtained from conventional feed analysis.

Effect of intrinsic characteristics on digestion kinetics

Plant species and maturity are the two most important sources of variation in digestion kinetics (Mertens, 1993a). Both the extent and rate of NDF digestion decrease with advancing maturity of grasses and legumes (Smith *et al*., 1972). Close positive correlation between the indigestible residue and growing days was observed in the data of Nousiainen *et al*. (2003a) for 27 grass silages (mixtures of timothy and meadow fescue) harvested across seven years at different stages of maturity. A similar negative relationship was observed between growing days and the rate of DNDF digestion estimated using equation 5 (Figure 2). Cone *et al*. (1999) also reported close ($R^2 > 0.90$) relationships between growing days and both the extent and rate of digestion of grass silage. Digestion rates of legumes are higher compared with grasses (Smith *et al*., 1972; Grenet, 1989; Van Soest, 1994) and the difference in digestion rates between red clover and timothy is much higher for stems than leaves (Rinne and Nykänen, 2000).

Digestion rates reported in the literature are highly variable between feeds, and surprisingly also within plant species. This raises the question whether these differences always reflect true differences in the digestion rate between feeds and dietary treatments and to what extent do the differences reflect the experimental techniques used. Smith *et al*. (1972) were the first to measure digestion kinetic parameters for a wide range of forage samples. The average DNDF digestion rate for grass samples was 0.096 h^{-1} and the range for early and late harvested samples varied from 0.140 to 0.053 h^{-1}. Although some grasses were harvested at a very early stage, the high values may also reflect the short incubation time (72 h) used to estimate the indigestible fraction. The average INDF concentration was 190 g kg^{-1} DM, which was more than 2-fold higher compared with that in primary growth grass silages (78 g kg^{-1} DM) harvested at various stages of growth (Nousiainen *et al*., 2004). Nousiainen *et al*. (2004) determined INDF by 12 d *in situ* incubations using nylon

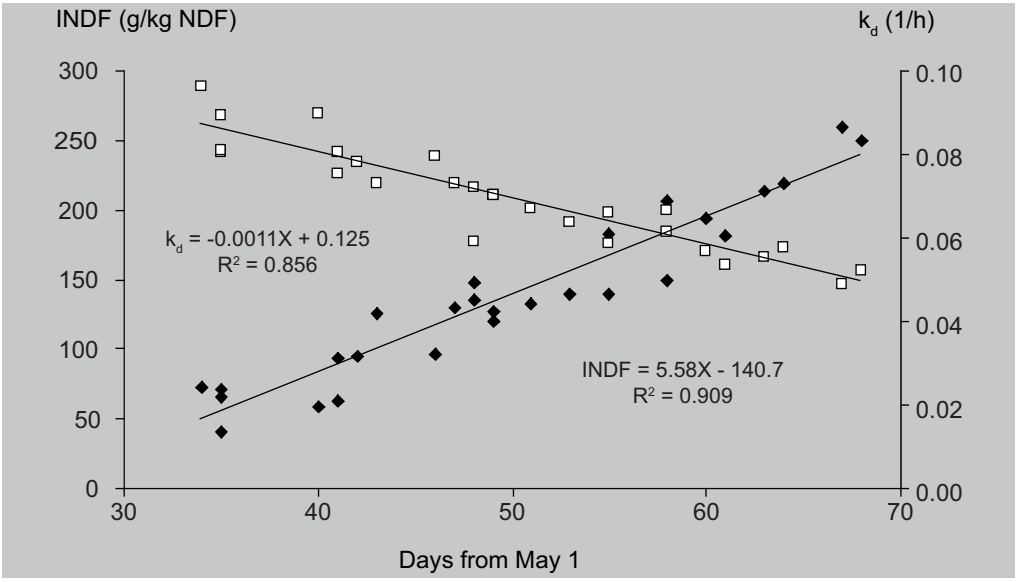

Figure 2. Effects of maturity of primary-growth timothy-meadow fescue silages on the proportion of indigestible NDF (g INDF/kg NDF) and the rate of NDF digestion. The values are adjusted for a random year effect. (Source: data from Nousiainen et al.*, 2003b).*

bags of a small pore size. The average DNDF digestibility of grasses calculated from the data of Smith *et al.* (1972) was 0.686, which was markedly lower than *in vivo* NDF digestibility (0.754) measured in sheep (Nousiainen *et al.*, 2004). The rate of digestion of silages estimated by equation 5 from *in vivo* DNDF digestibility was markedly lower (0.070 h^{-1}) than reported by Smith *et al.* (1972). Although the comparison of two different data sets is problematic, it is probable that the contrasting differences of digestion parameters are more likely related to methodological differences than a reflection of true differences in digestion kinetics.

Attempts to predict digestion kinetic parameters from chemical composition have been met with variable success. A close linear relationship between lignin and INDF contents for a diverse population of forage samples was reported by Smith *et al.* (1972) and Mertens (1973). The correlation between permanganate lignin and INDF concentrations in the data of Nousiainen *et al.* (2004) were also high both for primary growth and regrowth silages (0.86 and 0.91) but pooling this data together resulted in a weaker relationship (r = 0.61) suggesting that the association between lignin and INDF is not uniform. Regrowth silages contained more INDF than primary growth silages at the same lignin concentration. Furthermore, INDF estimated by *in situ* incubation predicted faecal NDF output (g NDF per kg DM intake) better than lignin (r = 0.91 vs. 0.79), and the relationship was more uniform between primary and regrowth silages with INDF than lignin. Satter *et al.* (1999) presented data demonstrating no relationship between lignin content and *in vitro* NDF degradability. We conclude that although lignin certainly plays a role in the cell wall degradation, and consequently is closely correlated with INDF concentration,

measurements of lignin cannot be used universally for the estimation of INDF concentration or potential NDF digestibility.

Predicting the rate of digestion from chemical parameters may be even less successful than predicting the extent of digestion. Smith *et al*. (1972) reported high correlations between the rate of cell wall digestion and some chemical measurements within forage species or type (grass vs. legume) but when the data was combined the relationships were much weaker. Weisbjerg *et al*. (2003) found using barley and whole crop wheat forages that both NDF and ADF, but not ADL, were significantly and negatively correlated with the rate of NDF digestion. Wilman *et al*. (1996) reported that the rate of cell wall digestion was negatively correlated (r = -0.81) with silage NDF concentration and proposed that leafier crops were digested more quickly. A similar inverse relationship between NDF concentration and the rate of DNDF digestion was observed for primary growth silages (r =-0.80), but not for regrowth silages (r =-0.14) in the data of Nousiainen *et al*. (2004). Sauvant *et al*. (1995) also showed based on a large data set that the rate of cell wall digestion is negatively related to the cell wall content of forages, but no such relationships were observed for concentrates. Mertens (1993a) suggested that the inverse relationship between NDF concentration and NDF digestion rate may be related to thickened cell walls which are less fragile to particle breakdown and microbial penetration. Although the concentration and digestion rate of NDF can be closely related for certain types of forages such as primary growth grasses, the relationship is not uniform across a wide range of feeds.

Effect of extrinsic characteristic on digestion kinetics

The intrinsic rate and extent of cell-wall digestion set the upper limit for the utilisation of forages by ruminants. Extrinsic factors are independent of intrinsic factors, and may down regulate or decrease the rate, such that the intrinsic rate is not achieved, if rumen conditions are not ideal. Dietary components have different effects on rumen microbes, and interactions between dietary components in rumen digestion can occur. In addition to accurate estimates of digestion kinetic parameters, the effects of various extrinsic factors on digestion kinetic parameters should be understood to predict digestibility properly by dynamic mechanistic rumen digestion models. For low quality forages, limitations in the rate and extent of digestion can be attributed to a deficiency in the supply of essential nutrients such as N, S or in some cases branched-chain VFA (Hoover, 1986). In contrast, in high producing ruminants fed mixed diets, the rate of cell wall digestion in particular can be strongly retarded by substrates which inhibit the growth of rumen cellulolytic bacteria. In the following sections we will briefly discuss extrinsic factors, which may influence the intrinsic digestion kinetic parameters of cell-wall carbohydrates. A detailed discussion of the possible mechanisms behind these is beyond the scope of this review.

Carbohydrate supplementation

Increasing the concentration of non-structural carbohydrates (mainly starch and sugars) in the diet has frequently been shown to decrease fibre digestion. Decreases in the rate of cell wall digestion with increased supply of non-structural carbohydrates has been attributed mainly to lower ruminal pH, because cellulolytic bacteria are more sensitive to low pH than those utilising starch (Hungate, 1966; Russel and Dombrowski, 1980). *In vitro* (Grant and Mertens, 1991) and

in situ data (Mould *et al*., 1983) suggest that rumen pH affect digestion kinetics in a biphasic manner. Above pH 6.2, the effects of pH on ruminal cell wall digestion are relatively small, but at a lower pH the effects are much stronger. Huhtanen and Jaakkola (1993) studied the effect of increased concentrate supplementation (barley + rapeseed meal) on cell wall digestion in cattle fed grass silage or barn dried hay as the basal forage. Total NDF digestibility decreased much more when the proportion of concentrate was increased from 0.50 to 0.75 than for increases from 0.25 to 0.50. Rumen pH decreased linearly from 6.43 at the lowest concentrate level to 6.21 and 6.03 with the medium and high concentrate diets, respectively. The mean rate of NDF digestion for the two forages determined by the rumen evacuation technique was depressed in a biphasic manner from 0.081 (low) to 0.075 (medium) and 0.047 (high), respectively. These results also suggest that a threshold pH for rumen cellulolysis is approximately 6.2, below which the degree of decrease in the rate of NDF digestion is much higher. The effect of increased concentrate on *in situ* NDF digestion rate was smaller than that observed by rumen evacuation, probably because the extent of *in situ* NDF digestion also tended to decrease. A reduced rate of NDF digestion estimated by rumen evacuation has been reported by Khalili and Huhtanen (1991) with sucrose supplements, by Stensig *et al*. (1998) with sucrose and starch and by Oba and Allen (2003) with dietary starch supplementation.

Lindberg (1981) studied the effects of oats fed proportionately at 0, 0.30 and 0.70 of diet DM on *in situ* digestion kinetics of forages. It was noticeable that increasing the amount of oats in the diet was associated with a decrease in both the rate and extent of NDF digestion. Similar effects were later reported by Huhtanen and Jaakkola (1994), who incubated six grasses in the rumen of cattle fed grass silage or barn dried hay with proportionally 0.25, 0.50 or 0.75 concentrates of diet DM. However, care should be exercised in interpreting these findings. It is possible that the longest incubation periods were too short to allow an accurate estimation of the extent of NDF digestion.

Grant and Mertens (1991) reported that the effect of rumen pH on *in vitro* cell wall digestion varied with substrate. Digestion of legumes was less sensitive to lower rumen pH than that of grasses. The results of *in situ* studies by Mould *et al*. (1983) and Huhtanen and Jaakkola (1994) indicate that digestion of low quality forages is influenced to a greater extent by increased concentrate supplementation.

Mould *et al*. (1983) differentiated the adverse effects of non-structural carbohydrates on cell-wall digestion between a ´pH effect´ and a ´carbohydrate effect´. The depression in cell wall digestion that could not be alleviated by increasing rumen pH with buffers was designated the ´carbohydrate effect´ and the depression related to low pH was designated as the ´pH effect´. When the supply of rapidly degradable substrates such as sugars and starch is excessive, the bacteria using these substrates will predominate in the rumen. Under these circumstances cell wall digestion could be impeded due to high acid production or the use of limiting nutrients by these bacteria. Although the evidence suggests that the depression in cell-wall digestion is associated with reduced rumen pH, there is little evidence that it is the sole causative factor (Mertens, 1993a). Intraruminal infusion studies (Rooke *et al*., 1987; Huhtanen, 1987) indicated that cell wall digestion can be depressed by a continuous supply of rapidly degradable carbohydrates without decreasing rumen pH. Mertens and Loften (1980) observed that when pH was maintained at 6.8, digestion rate of

forage NDF was reduced slightly and the lag time increased markedly when starch was added *in vitro*. Reduced cell wall digestion with continuous infusion of sucrose was associated with lower particle-associated enzyme activities in rumen digesta (Huhtanen and Khalili, 1992). Studies using continuous cultures allowing for independent changes in pH and level of rapidly degradable carbohydrates showed that the level of the rapidly degradable carbohydrates was the most important for fibre digestibility (Weisbjerg *et al*., 1999). *In vitro* work (Groleau and Forsberg, 1981; Williams and Withers, 1982) has indicated that the activity of cell-wall degrading enzymes depends on the carbon source.

Protein supplementation

When N supply becomes limiting, cell-wall digestion is retarded. Therefore it is essential that N supply is not a limiting factor when intrinsic digestion parameters are determined. Hoover (1986) suggested a minimum ammonia concentration of 3.6 mmol L^{-1} when dietary crude protein (CP) concentration exceeds 60 g kg^{-1} DM. Ellis *et al*. (1999) reported that protein supplementation increases the rate of digestion of cell-wall carbohydrates of forages which contain less than 80 g CP kg^{-1} DM. Mertens (1993a) postulated that the minimum amount of available N depends on the digestibility of forages, and is relatively higher for highly digestible forages. The concentration of dietary N needed to optimise cell-wall digestion is also a function of ruminal protein degradability (Ørskov, 1982). Positive effects of protein level on cell-wall digestion in dairy cows were reported by Oldham (1984) at a much higher level than the suggested minimum for optimal digestion. An increase of 7.3 g kg^{-1} in NDF digestion per 10 g kg^{-1} DM increase in dietary CP was estimated from a data set (N = 182) of studies conducted in lactating dairy cows (Huhtanen *et al*., unpublished). Dietary CP was increased by replacing energy supplements with protein supplements such as rapeseed, soybean and fish meals. However, the mechanisms of protein responses in dairy cows are not completely clear, and the effects may partly be mediated through changes in the intrinsic characteristics of cell walls and partly through the effects of protein on microbial activity in the rumen. Improved digestibility of DNDF with protein supplementation reported by Shingfield *et al*. (2003) suggests that an increased availability of amino acids in the rumen improved cell wall digestion that may have been mediated through increases in the rate of digestion. However, the possible effects of reduced starch content in the diet can not be ruled out as a mechanism for improved digestion rate with increased dietary CP. The singular effects of degradable protein can be determined using urea as a N source. Weisbjerg *et al*. (1998) added 260 g urea d^{-1} per cow to a ration highly deficient in rumen degradable protein and increased dietary CP from 112 to 144 g kg^{-1} DM resulting in an enhanced rate of DNDF degradation from 0.019 to 0.031 h^{-1}, although *ad libitum* feed intake increased also considerably.

Fat supplementation

Supplementation of a diet with fats or fatty acids can affect ruminal metabolism and reduce fibre digestion. This is due to the toxic effects of fatty acids on rumen bacteria, particularly unsaturated and to a lesser extent medium chain fatty acids which can reduce fibre digestion (Weisbjerg and Børsting, 1989; Demeyer and Van Nevel, 1995; Doreau and Chilliard, 1997), but these negative effects are not always seen (Ueda *et al*., 2003). Use of more rumen inert (protected) fat sources such as saturated fatty acids or calcium soaps of unsaturated fatty acids reduce negative effects of

the fat supplementation on rumen metabolism. Generally negative effects on fibre digestibility will not be manifested until fat supplementation exceeds 40-50 g kg^{-1} DM, but this limit depend on whether the fat is inert, on fatty acid concentration and composition of the basal ration and on the physical structure of the diet (Doreau and Ferlay, 1994; Lewis *et al*., 1999).

The results of Tesfa (1992, 1993) indicate that high levels of rapeseed oil (67 g kg^{-1} DM) decreased the rate of NDF digestion markedly when determined by *in situ* or rumen evacuation techniques. Reduced particle-associated enzyme activities in rumen digesta and undigested *in situ* residues suggested that decreased cell wall digestion was associated with the adverse effects of oil supplementation on the activity of rumen cellulolytic bacteria rather than from the oil coating fibre particles.

Due to the high energy content, isoenergetic supplements of fat can be used to replace large amounts of rapidly degrading carbohydrates (starch) in concentrates. Therefore fat with a low iodine value can have a positive effect when added on an isoenergetic basis, due to the substitution with more problematic starch.

Since fat supplementation can alter rumen metabolism, it could also be expected to affect rumen passage kinetics. Although adverse effects have been seen in some studies, Doreau and Ferlay (1995) concluded from an analysis of 18 studies, that neither liquid or solid phase turnover was affected by fat supplementation.

Feeding level

Diet digestibility decreases with increased feed intake. Reduced digestibility has mainly been attributed to decreased rumen residence time allowing less complete digestion of DNDF. The effects of feeding level on the rate of NDF digestion have not been extensively studied. Reduced rates of NDF digestion have been reported by Staples *et al.* (1984), Robinson *et al.* (1987) and Okine and Mathison (1991) when DM intake increased. Staples *et al.* (1984) used an *in situ* technique, while the others used rumen evacuations. One reason for the adverse effect of feed intake on cell-wall digestion is that rumen VFA concentrations increase, with a concomitant decrease in rumen pH (Tamminga and van Vuuren, 1988; Volden, 1999), to which cellulolytic bacteria are sensitive. Huhtanen *et al.* (1995) fed growing cattle at 8.5 or 17.0 g DM kg^{-1} LW and reported that feeding level had no effect on rate of NDF digestion as estimated by rumen evacuation. The estimates based on *in situ* data suggested a trend towards a decrease in the extent, but an increase in the rate of digestion with increased feed intake. The decrease in total NDF digestibility from 0.758 to 0.707 could be entirely due to higher passage rates. Indirect comparison of digestion rates estimated by rumen evacuation data in growing cattle and dairy cows fed similar diets (Rinne *et al*., 1997, 2002) suggest a reduced rate of digestion with increased feeding level. In both studies animals were fed four grass silages harvested at one week intervals. The silages had similar differences in INDF concentration (cattle: 143-228; cow: 149-217 g kg^{-1} NDF) and cows and young cattle were both fed the same proportion (0.30) of a similar concentrate (cereal grain and rapeseed meal). Feeding level was markedly higher for dairy cows than for growing cattle (32 vs. 17 g DM kg^{-1} LW). Estimated rates of DNDF digestion were clearly higher in growing cattle than in cows (0.073 vs. 0.056 h^{-1}). Digestion parameters of the silage estimated by the *in situ*

method were similar, but rumen pH was clearly lower in cows than cattle fed comparable diets, which may be the reason for the lower digestion rate observed in cows. The results of Llano and DePeters (1985), Huhtanen *et al*. (1995) and Volden (1999) suggest that reduced OM digestibility with increased DM intake can almost entirely be attributed to lower NDF digestibility. However, for some diet types e.g. rations rich in slowly degradable maize starch, an increase in DM intake can also significantly reduce the digestibility of cell solubles (Colucci *et al*., 1982).

Validity of digestion kinetic methods

Information about the rate and extent of cell wall digestion has been increased by the use of *in situ* and *in vitro* techniques. However, *in vivo* validation of the results has seldom been carried out. Due to the lack of reliable and widely accepted reference methods, the merits and demerits of different digestion kinetic methods can not be verified. Ellis *et al*. (1994) suggested that the *in situ* method is preferable because aspects of rumen environment are more faithfully simulated. It has further been argued that the *in situ* method also measures the combined effects of both the animal and the diet on digestion, which can be considered as a disadvantage in the determination of intrinsic digestion kinetic parameters. The advantages related to the applicability of *in vitro* and *in situ* methods with respect to obtaining meaningful kinetic data have been discussed in detail (Mertens 1993a, 1993b). Mertens presented several critical aspects of the *in situ* method: kinetic results may be biased because of non steady-state conditions in the rumen (1), suboptimal conditions in the rumen may put an upper limit on the rates (2) and inflow and outflow of particles to the bag (3). These aspects are probably more critical for the determination of the intrinsic rate of digestion than for the determination of the extent of digestion. The close relationship between *in vivo* digestibility and the potential extent of digestion (Nousiainen *et al*., 2003b) suggests that using prolonged incubations and bags with a small pore size may allow the extent of NDF digestion to be accurately measured.

Digestion rates determined by *in situ* incubation have been lower than values derived from rumen evacuation in studies conducted at different laboratories (Aitchison *et al*., 1986; Tamminga *et al*., 1989; Huhtanen and Jaakkola, 1993; Rinne *et al*., 2002). These findings suggest that normal microbial colonization of samples within the bags was not achieved and/or that conditions in the bag were sub-optimal. Meyer and Mackie (1986) showed that the bacterial population inside the bags was lower than in the surrounding digesta, particularly for the cellulolytic bacterial populations. Lower fibrolytic activities in particle-associated microbes in bag residues than in rumen digesta (Huhtanen and Khalili, 1992; Nozière and Michalet-Doreau, 1996) is consistent with lower microbial numbers within the bags. The pH in the bags has also been lower than in rumen digesta (Nozière and Michalet-Doreau, 2000), which points towards the sub-optimal conditions within the bag. The differences in microbial activity may be explained by a shorter residence time of feed particles in bags compared with rumen digesta, the lack of mastication of forage particles placed in the bags or limiting conditions within the local bag environment (Nozière and Michalet-Doreau, 2000). The highest particle-associated enzyme activities within the bags were proportionally less than 0.50 of those found in rumen digesta (Huhtanen *et al*., 1998) indicating that colonization of the cell degrading bacteria is constrained within the bags. In their study particle-associated enzyme activities in bag residues and NDF disappearance were greatly reduced with smaller pore size and/or smaller open surface area of the bags. Disappearance

of NDF from bags of different cloth types incubated for 6, 12, 24 and 96 h was closely related to the logarithmic transformed cumulative area under enzyme activity curves. This suggests that enzyme activity, rather than intrinsic characteristics of forages, limit cell wall digestion of feeds incubated in nylon bags.

If the rate of NDF digestion *in situ* is constrained by reduced microbial colonization and/or low pH in the bags, digestion kinetics determined *in vitro* may describe the intrinsic digestion kinetics more accurately than the *in situ* method. Cone *et al.* (1998) reported that *in situ* rates of NDF and OM digestion were closely related to the rate of digestion estimated from the second sub-curve of the total gas production curve, which corresponds to the degradation of non-soluble OM (Cone *et al.*, 1997). However, the estimated rates were much higher with the gas production than with the *in situ* technique. The relationship between digestion rates estimated from gas production profiles or by the *in situ* technique was weak for hays (Khazaal *et al.*, 1993) and straws (Blümmel and Ørskov, 1993). The gas production profiles were fitted to a single phase exponential model, and the gas production from the soluble fraction is included which is not the case for the *in situ* method. Firkins *et al.* (1998) suggested that the accuracy of determination of the rates of gas production can never be greater than the method of the verification (*in situ* or *in vitro* substrate depletion kinetics). However, comparing different kinetic methods (*in vitro, in situ*) with each other may be of little value before the accuracy of these methods has been improved.

Huhtanen *et al.* (2001) measured gas production kinetics of NDF isolated from 15 samples of grass silages harvested at different stages of maturity. The first-order NDF digestion rate estimated from gas production profiles was closely related with the rate of NDF digestion derived from *in vivo* measurements (see equation 5). However, the rate of NDF digestion estimated for a sub-set of six of the silages by ruminal *in situ* incubation was clearly underestimated compared with *in vivo* measures, but a strong linear relationship between the estimates based on *in situ* and gas production kinetics was observed (Figure 3).

Passage kinetics

Microbial digestion of cell walls is a relatively slow process. To achieve effective cell wall digestion, ruminant animals have developed large fermentation chambers in the fore-stomach, where they retain feed particles substantially longer than fluids. A long retention time in the reticulorumen improves the utilization of cell walls but it may restrict feed intake. The ruminal digestibility is determined by the rates of digestion and passage, i.e. digestion and passage are considered to be competitive processes. Unlike digestion kinetics that can be measured by *in vitro* or *in situ* methods, passage kinetics must be determined *in vivo* due to the interaction between diets and animals. Methods for the estimation of passage kinetics are laborious and expensive. The advantages and disadvantages of different markers and mathematical models applied are still debatable, such that the biological interpretation of estimated parameters is often uncertain and the parameter values are often erroneously used in rumen models to estimate digestibility. Different aspects related to the techniques used to estimate digesta passage have been the subject of numerous reviews (Lechner-Doll *et al.*, 1991; Faichney, 1993; Mertens, 1993a; Ellis *et al.*, 1994, 1999). This section will focus on the aspects of digesta passage kinetics relevant to the estimation of kinetic data for

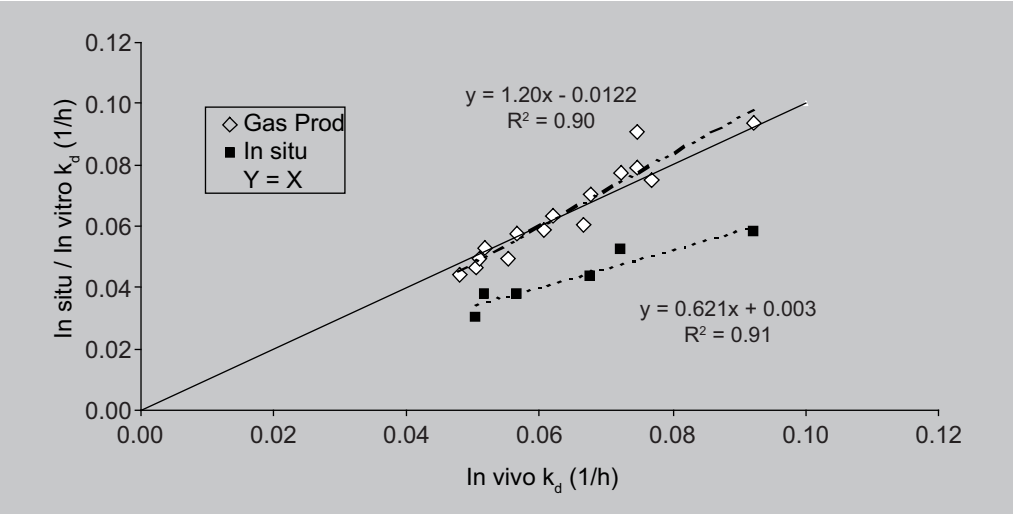

Figure 3. Digestion rate (k_d) estimated from in vivo data or by using in vitro gas production and in situ techniques (Data from Huhtanen et al.*, 2001; Nousiainen* et al.*, 2004; unpublished in situ data).*

mechanistic rumen models. Simulation results presented in Table 4 highlight the importance of accurate determinations of rumen residence time on the predictions of nutrient supply.

Methodology

Markers

In parallel with digestion kinetic methodology, the progress in developing an effective and accurate passage kinetic methodology has been retarded by the lack of a reliable, non-laborious and simple reference method to validate and interpret the kinetic data.

Table 4. The effects of rumen residence time on digestibility, rumen NDF pool and microbial N flow simulated by the Nordic dairy cow model (Source: Danfær et al.*, 2005a).*

	Rumen residence time (h)				
	30	**35**	**40**	**45**	**50**
Digestibility					
OM	0.657	0.676	0.691	0.703	0.713
NDF	0.608	0.637	0.659	0.678	0.693
DNDF	0.730	0.764	0.791	0.814	0.832
NDF pool (kg)	6.01	6.59	7.11	7.59	8.03
Microbial N flow (g d^{-1})	200	205	210	213	217
INDF 100 g kg^{-1} DM, DNDF 500 g kg^{-1} DM, k_d of DNDF 0.05 h^{-1}					

Different markers, sampling procedures and methods of compartmental analysis have been applied, but the biological interpretation of compartmental analysis is debatable.

To estimate digesta passage rate, a limited amount of the marker is administered, usually as a single pulse-dose, followed by digesta sampling. Various external markers have been used to describe passage kinetics. Ideal markers are indigestible, i.e. have no effect or be little affected by the microbial population, and must be associated with undigested nutrients, or flow through the digestive tract at an identical rate, and do not separate from the respective labelled fraction (Kotb and Luckey, 1972; Ellis *et al*., 1994). Probably none of the current passage kinetic markers satisfy all these criteria. The chromium mordanted fibre method, as described by Udén *et al*. (1980), has been criticized because it renders the fibre indigestible and tends to increase particle density (Ehle *et al*., 1984), which may increase passage rate. In contrast to the generally observed relationship between density and passage rate (Lechner-Doll *et al*., 1991), Lirette and Milligan (1989) reported a shorter total mean retention time (TMRT) for particles labelled at a low compared with high levels of Cr (0.2 vs. 5 g kg^{-1} DM), probably because the lower degree of mordant had less of an effect on the digestibility of labelled particles. In their study, TMRT was 13-14 h longer for 10 mm compared with 1-2 mm particles, demonstrating the importance of particle size in passage kinetic determinations. The particle size of the labelled feed should be similar to that of unlabelled feed.

Rare earths are probably the most commonly used passage kinetic markers. Rare earths are indigestible and are resistant to replacement from feed residues within the normal pH range in the rumen (Ellis *et al*., 1994). Rare earths have been criticised since they migrate to rumen fluid (Beauchemin and Buchanan-Smith, 1989; Combs *et al*., 1992) and are preferentially bound to small rather than large particles (Siddons *et al*., 1985). A longer CMRT of Cr-mordanted fibre compared with Yb-labelled fibre (Beauchemin and Buchanan-Smith, 1989; Huhtanen and Kukkonen, 1995) suggests that at least one of these markers does not behave as an ideal marker. Removing unbound or loosely-bound rare earths by washing with a mild acid solution may solve the problems related to the dissociation of the marker from low affinity binding sites in labelled feed particles to rumen microbes and liquid phase or from large to small particles (Ellis *et al*., 1994), which have a faster passage rates. The validity of the marker system may be tested by comparing the marker retention time to that estimated using the rumen evacuation technique for an internal marker naturally included in the feed such as INDF or lignin. Ellis *et al*. (2002) reported a similar CMRT estimated from the passage kinetics of rare earths or from the turnover of INDF from the rumen. They postulated that migration of rare earths from labelled particles, which has sometimes been observed, is probably a result of applying rare earths in excess of their binding capacity and failure to remove excess or unbound rare earths. Huhtanen and Kukkonen (1995) compared the CMRT estimated from duodenal Yb and Cr concentrations to that estimated by rumen evacuation technique and INDF turnover. A mean CMRT of 67, 57 and 63 h was calculated for Cr-mordanted fibre, Yb-labelled fibre and INDF, respectively, in cattle fed at two levels of intake. These results indicate that Cr slightly overestimated, and Yb underestimated CMRT based on ruminal INDF turnover. Lund (2002) also found that Yb-labelled fibre underestimated CMRT compared with INDF and rumen evacuation, particularly for diets with the highest CMRT of INDF. Earlier conclusions (e.g. Tamminga *et al*., 1989; Huhtanen and Kukkonen, 1995) that Cr-mordanted fibre underestimates rumen retention time (overestimates

the passage rate) were mainly due to the estimation of passage rate from the descending faecal marker excretion curve and ignoring the ascending phase in marker concentrations.

Intrinsically labelled plant cell walls should be ideal markers because they undoubtedly flow with undigested feed residues. The problem of internal markers such as ^{13}C and ^{14}C is that the label is incorporated into both the digestible and indigestible cell wall fractions. Digestible components should therefore be removed before dosing (Smith, 1989), or alternatively the marker concentration could be measured in the INDF fraction. Intrinsically labelled forage ADF-bound ^{15}N was used as a passage kinetic marker by Huhtanen and Hristov (2001). Ahvenjärvi *et al.* (2004) compared ADF-^{15}N in grass silage to Cr-mordanted and Yb-labelled silage as passage kinetic markers in dairy cows. They observed that the CMRT and total mean residence time (TMRT) were similar for ADF^{15}N and Cr-mordanted but shorter for Yb. The use of intrinsic markers is too laborious and expensive for routine use, but it may be a useful tool for evaluating the reliability of external markers.

Compartmental analysis

Similarly as for digestion kinetics, various mathematical models have been proposed for the estimation of passage kinetics. The earliest compartmental model to describe digesta flow based on faecal marker excretion data was described by Blaxter *et al.* (1956). The model consisted of two sequential age-independent mixing compartments with a discrete time delay. Grovum and Williams (1973) proposed that the two sequential compartments represent the retention of feed particles in the rumen and the caecum-proximal colon. Matis (1972) proposed a two compartmental model with sequential age-dependent and age-independent compartments and a time delay. He assigned an age-dependent distribution of residence times to the faster compartment, which describes processes such as hydration, microbial colonization and fragmentation of feed particles by rumination. The flow from the second, age-independent compartment is described by simple first-order kinetics. Details of the age-dependent models are described by Pond *et al.* (1988) and Ellis *et al.* (1994). The models of Dhanoa *et al.* (1985) and France *et al.* (1985) also describe age-dependent processes but use different mathematical approaches.

Estimates of rumen passage kinetics can be verified by the rumen evacuation technique or by slaughter studies. The mean residence time (MRT) in each segment of the digestive tract using indigestible NDF as a marker is calculated as: MRT (h) = INDF (g) in the segment / INDF intake (g/h). Paloheimo and Mäkelä (1959) used this method to estimate turnover time of lignin in different sections of the digestive tract of cows. Residence time in different sections of the digestive tract can also be estimated by marker dosing and sampling digesta from different sites of the digestive tract. Only small differences in the CMRT estimated from duodenal or faecal samples have been observed in cattle when different external markers (Pond *et al.*, 1988; Huhtanen and Kukkonen, 1995; Wylie *et al.*, 2000; Lund, 2002) or an internal marker (Huhtanen and Hristov, 2001) were used. These observations suggest that most of the residence time in the first compartment is pre-duodenal. Ellis *et al.* (2002) proposed that the proportion of the total compartmental residence time due to the mixing flow in the rumen is relatively constant (in order of 0.9), and that CMRT in the rumen could be predicted from faecal marker profiles. Indeed, faecal sampling may be even more accurate in predicting pre-duodenal CMRT due to the inability of collecting representative

samples of rumen digesta and problems in obtaining representative samples of duodenal or ileal digesta. Dosing particle markers in the abomasum (Wylie *et al*., 2000) or duodenum (Huhtanen and Kukkonen, 1995; Mambrini and Peyraud, 1997) and sampling faeces also indicated that post-ruminal residence time in the mixing compartments is relatively short, representing less than 0.10 of total CMRT estimated from faecal sampling. In the slaughter study (Paloheimo and Mäkelä, 1959), the proportion of retention in the hind-gut of that in the total tract for lignin was 0.10 which is entirely consistent with the marker kinetic data.

Interpretation of marker kinetic data obtained from duodenal sampling supports the suggestion of Hungate (1966), who proposed that there are two different compartments: a rumination pool of large particles and a passage pool of small particles, where the passage of initially large particles from the rumen is a result of two sequential first-order processes. Actually, the models of Matis (1972) and his co-workers (Pond *et al*., 1988; Ellis *et al*., 1994) proposed an age-dependency to the rumination pool of the large particles. Faichney (1986) seriously questioned this approach. First, the model does not take into account the entry of small particles produced by chewing during eating, and secondly, the abomasum and caecum/proximal colon act as mixing compartments. However, as previously discussed the contribution of post-duodenal segments to total CMRT is relatively small, at least in cattle. When faecal particles labelled with rare earths were dosed into the rumen followed by duodenal or faecal sampling, the CMRT in the age-dependent compartment was about 9 h and not different between the sampling sites (Wylie *et al*., 2000). Dosing labelled faecal particles into the abomasum, and estimating total CMRT from duodenal and faecal sampling indicated residence times of 1.1 and 3.0 h in the abomasum and hind-gut, respectively, indicating that these sites contribute relatively little to the total residence time in mixing compartments.

The models with gamma time-dependency have improved the fit of the data compared with the two-compartment model with first-order passage from both compartments (Pond *et al*., 1988; Ellis *et al*., 1994; Huhtanen and Kukkonen, 1995; Lund, 2002). However, this does not necessarily imply that these models describe the distribution of the total retention time in different segments of the gastrointestinal tract any better than other models. Increasing the degree of age-dependency in the first compartment changes the partitioning between the time delay and the age-dependent residence time. However, estimates of time delay are more consistent with observed data (first appearance of marker at the sampling site) with age-dependent models (Pond *et al*., 1988; Huhtanen and Kukkonen, 1995). Conversely, estimates of CMRT should be more consistent with actual data, but validation is more difficult. In the study of Huhtanen and Kukkonen (1995), CMRT estimated from duodenal sampling was 52, 52, 56 and 60 h with increasing age-dependency in the first-compartment. The last two values are in better agreement with INDF turnover time estimated by rumen evacuation (63 h).

Most of the experimental data indicate that the passage of feed particles in ruminants is a multi-compartmental process. When marker excretion data is fitted with a two-compartment model, only the residence time in two compartments (or aggregated compartments) can be described (Mertens, 1993a). However, if the passage kinetic parameters are estimated simultaneously from duodenal and faecal sampling, fairly accurate representations of the residence time of particles in different compartments may be obtained. We estimated the passage kinetic parameters from

simulated duodenal and faecal marker concentration data using passage kinetic models with increasing age-dependency in the first compartment. The following residence times in different sections of digestive tract were used: rumen large particle (lag-rumination) pool 10 h (with an exponential or 2nd order gamma distribution of residence times), rumen pool of small particles 25 h (exponential distribution of residence times), omasum 4 h (mixing flow), abomasum and small intestine 4 h (tubular flow), caecum and proximal colon 5 h (mixing flow), distal colon 4 h (tubular flow). The parameter values estimated by the best fit model of series of the models with age-dependency in the first compartment are shown in Table 5. When the rumen lag-rumination (non-escapable) pool had gamma two age-dependency, G_3G_1 model (see Ellis *et al.*, 1994) resulted in the best fit of simulated duodenal marker concentration, and the G_4G_1 model was the best for faecal sampling data. Similarly, Lund (2002) found that a higher order gamma function was needed to fit faecal sample data compared with duodenal measurements, suggesting the existence of an additional post duodenal compartment (Huhtanen and Hristov, 2001). The best fit models estimated the ´true´ time delay and CMRT correctly. Interestingly, the residence times in the omasum (duodenal sampling) and omasum + caecum + proximal colon (faecal sampling) were realised as an increase in the residence time in the lag-rumination compartment. This suggests that the model does not correctly describe the biological processes of digesta passage. However, this does not mean the parameter values estimated by the passage models would be of little value for mechanistic rumen digestion models. The best fit models estimated accurately both the pre-duodenal and total residence time in the mixing compartments, and consequently the residence time in the post-ruminal mixing compartments was correctly estimated by difference. When more than two mixing compartments exist and/or the residence time in the lag-rumination pool (the first compartment) is age-dependent, a two-compartment model with an exponential distribution of residence times also provides an adequate description of the data. When the parameters are estimated by the two-compartment model with no time dependency, the TMRT may be correctly estimated, but the distribution of the TMRT between the time delay and CMRT can be seriously biased. This bias can result in considerable errors in estimates of cell wall digestibility.

Particle dynamics

Ruminant animals have developed a strategy to take full advantage of digestible energy in forages by selective retention of digesta particles in the rumen. The large particles and particles containing a high proportion of digestible material are selectively retained in the rumen, whereas the particles containing less digestible material have a higher probability of escaping from the rumen. Several excellent reviews of particle kinetics have been published (Faichney, 1986; Kennedy and Murphy, 1988; Kennedy and Doyle, 1993; Murphy and Kennedy, 1993). The following discussion will briefly encompass the mechanisms of selective retention, and the determination of kinetic parameters related to the release of feed particles from the rumen lag-rumination compartment to the escape pool.

Selective retention of feed particles in the rumen has been demonstrated by various techniques. The passage rate of feed particles from the rumen is inversely related to particle size (Poppi *et al.*, 1980; McLeod and Minson, 1988). Many authors have suggested that the critical particle size is approximately 1-2 mm because only a small proportion of particles appearing in faeces are retained on these screens. The distribution of particle length and width in rumen contents and faeces does

Table 5. Mean compartmental residence time and time delay estimated from synthetic marker excretion data by using the models with increasing time dependency in the first compartment (Source: see Pond et al.*, 1988).*

Sampling site	Model	$CMRT_1$	$CMRT_2$	CMRT	TD	TMRT	EMS
Duodenum [D]	G_1G_1	13.3	20.0	33.3	4.7	38.0	32.7
	G_2G_1	12.6	24.0	36.6	2.1	38.7	2.5
	G_3G_1	**14.0**	**25.1**	**39.0**	**0.0**	**39.0**	**0.0**
	G_4G_1	**13.2**	**27.0**	**40.2**	**0.0**	**40.2**	**17.0**
Rectum [R]	G_1G_1	15.4	20.1	35.5	16.4	51.9	52.5
	G_2G_1	15.9	22.8	38.8	12.7	51.4	6.8
	G_3G_1	17.4	24.3	41.7	10.1	51.8	0.8
	G_4G_1	19.0	25.1	44.1	8.0	52.1	0.0
Difference (R-D)[1]		5.1	0.0	5.1	8.0	13.0	

$CMRT_1$ = Mean residence time in the first mixing compartment (lag—rumination pool, $CMRT_2$ = Mean residence time in the slower turnover compartment, CMRT = Total mean compartmental residence time, TD = Time delay, TMRT = Total mean retention time, EMS = Error mean square).
The ´true´ residence times in the different segments of gastrointestinal tract were: rumen large particle (lag-rumination) pool 10 h (with an exponential or 2nd order gamma distribution of residence times), rumen pool of small particles 25 h (exponential distribution of residence times), omasum 4 h (mixing flow), abomasum and small intestine 4 h (tubular flow), caecum and proximal colon 5 h (mixing flow), distal colon 4 h (tubular flow).
[1] Calculated for the best fit models

not provide evidence supporting the critical particle size controlling the flow of particles from the rumen of cows fed grass silage (Nørgaard and Sehic, 2003). It is more likely that the probability of particles to leave the rumen decreases with increases in particle length and width. The concept of critical particle size has been questioned since a large proportion of rumen DM and particulate matter is below the suggested critical size (Ulyatt *et al.*, 1986; Lechner-Doll *et al.*, 1991). Lechner-Doll *et al.* (1991) postulated that specific gravity was twice as important as particle length in determining the likelihood of particles escaping the reticulo-rumen. Sutherland (1988) suggested that particles separate into those having buoyant properties attained via entrapped fermentation gases (newly ingested particles) and those having sedimentation properties after they have been depleted in fermentable substrates. Sutherland (1988) separated ruminal particles according to their buoyancy with warm artificial saline. Hristov *et al.* (2003) used a similar approach and observed that the sedimenting particles contained more INDF than the buoyant particles.

The passage rate estimated by rumen evacuation has been faster for INDF compared with DNDF for various diets (Tamminga *et al.*, 1989; Huhtanen and Jaakkola, 1993; Lund, 2002; Oba and Allen, 2003) demonstrating that digestible material is selectively retained in the rumen, in spite of the fact that digestible and indigestible fractions are contained in the same particles. Despite extensive efforts, the mechanisms controlling the separation and outflow of particles from the reticulo-rumen have not been unequivocally elucidated. Particle size reduction as a result of chewing and increase in specific gravity as a result of reduced fermentation activity occur

simultaneously with increased time after ingestion as indicated by a close negative correlation between particle size and specific gravity (Evans *et al*., 1973; Hooper and Welch, 1985). Microbial degradation facilitates particle breakdown during mastication by increasing particle fragility (Kennedy and Doyle, 1993). A close positive relationship between potential NDF digestibility and the size of particles (Ahvenjärvi *et al*., 2001) also supports the view that the density and size of particles are closely correlated. Gas production from an active fermentation decreases when fermentable substrates become depleted by increased residence time in the rumen. Because the particle size of digesta decreases concomitantly with increased residence time, it is difficult to interpret which mechanism, particle size or specific gravity, is more important in regulating the outflow of feed particles.

Selective retention of feed particles in the rumen is also evident from duodenal marker excretion curves (Pond *et al*., 1988; Ellis *et al*., 1994; Huhtanen and Hristov, 2001; Lund, 2002) which clearly indicate an ascending phase, which challenges the interpretation of the rumen as a single compartment system, where the probability of particles to escape is a random process. Whatever the mechanism underlying selective retention of feed particles, the process of selective retention should be incorporated into mechanistic dynamic rumen models to accurately predict cell wall digestibility. The total residence time in the reticulo-rumen fermentation compartments may be estimated by the rumen evacuation method or use of appropriate markers and compartmental models. With marker techniques, interpretation of rate constants related to specific compartments is difficult and highly dependent on the choice of model used. Information of residence time in the total tract may be more useful which is also less affected by the configuration of the model. As suggested by Ellis *et al.* (2002), the residence time in pre-duodenal fermentation compartments could be accurately estimated from faecal marker concentration data using appropriate kinetic models. However, the distribution of residence time between the two compartments in the forestomach (lag-rumination pool, escape pool) still remains debatable.

Kennedy and Doyle (1993) discussed the methods for measuring particle kinetics. One method is to estimate the decline of particle load by complete emptying of the rumen and based on the assumption of a linear or exponential decline, rate constants can be calculated. However, because the large particle load also disappears by digestion, the decline for indigestible fraction of the particle load should also be estimated to describe the rate of particle breakdown correctly. In the second method, the particle comminution rate is calculated from steady-state kinetics as [(input – escape (g h^{-1})) / load (g)]. The approach requires rumen evacuation data and an estimation of the large particle content of ingested feed. This method also requires that an allowance is made for digestion. Several marker techniques have been used to estimate the comminution rate of feed particles. The rate of particle comminution can be estimated by difference of ruminal or total retention time between the large labelled feed particles and faecal particles. Using this approach, very high comminution rates (0.1-0.3 h^{-1}) of feed particles have been reported by Bowman *et al.* (1991) and Cherney *et al.* (1991). Ellis *et al.* (1999) estimated residence time in the lag-rumination pool by fitting two compartmental models with age-dependency in the first compartment to marker data and concluded that the mean residence time in the lag-rumination pool is unaffected by dietary INDF level being relatively constant at around10 h.

Poppi *et al.* (2001) suggested an alternative interpretation of marker kinetics in the rumen, namely a raft model. Their reversible flow model based on marker kinetics data from dorsal and ventral rumen digesta had the following features: a relatively slow age-dependent transfer from the raft pool to the ventral rumen pool and a very rapid first-order exit from the ventral rumen-reticulum. It is important to note that the sequence of the rate constants: (slow raft, fast small particle) differ from earlier particle kinetic models (fast large particle, slow small particle). The high proportion of total rumen DM in the raft (0.75 – 0.89) was consistent with the escape from the raft being the rate limiting step. The proportion of raft material to total rumen digesta appears to be related to feed intake. The proportion of raft in total digesta increased from 0.42 to 0.95 when DM intake increased from 6 to 24 kg/d (Robinson *et al.*, 1987).

In animals fed *ad libitum,* the high proportion of raft and absence of a distinct mat and liquid phases may prevent free particle movement by sedimentation and flotation. Under these circumstances the capacity of the mat to entrap potentially escapable small particles may be an important mechanism to maximise fibre digestibility. A similar particle size distribution and potential NDF digestibility within each sieve size in the dorsal and ventral sacs and reticulum (Ahvenjärvi *et al.*, 2001) indicates that flotation and sedimentation may not be the main mechanisms influencing the escape potential of digesta particles as suggested by Sutherland (1988). Observations of the particle size distribution and potential NDF digestibility within each sieve size is consistent with the solid mat filling both the dorsal and ventral rumen. The high proportion (>0.50) of large particles (>2.5 mm) in rumen particulate OM in the dorsal and ventral sacs suggest that digesta passage is more limited by the release of particles from the raft pool into the escape pool. The results from a recalculation of data from Rinne *et al.* (2002) are also consistent with the raft model concept. The mean residence time of the large particle pool estimated as [(input – flow) / load] by making an allowance for digestion was on average 28 h for the four grass silage based diets. This estimate is almost three times higher than the residence time assigned to the lag-rumination pool based on two pool models with an age-dependent and age-independent residence time (Ellis *et al.*, 1999). The corresponding total rumen INDF residence time estimated by rumen evacuation was 41 h. When the entrapment of small particles by the mat is taken into account, the results can also be interpreted by the raft model, i.e. the retention time in the lag-rumination (raft) compartment was markedly longer than that in small particle escape compartment. The large particles (>2.5 mm) comprised proportionally 0.49 of particles in the rumen. These observations are consistent with those of Bruining *et al.* (1998) who found that rumen digesta contained more large particles than small particles. In their study the rate of particle comminution determined by steady-state kinetics ranged from 0.034 h^{-1} (grass silage based diet) to 0.049 h^{-1} (lucerne silage based diet).

The sequence of rumen compartments is difficult to determine from duodenal marker profiles. Assuming that the two rumen compartments consist of two sequential exponential pools, the order of the pools (fast-slow vs. slow-fast) has no effect on the marker profile. If the raft pool is gamma age-dependent, the marker profile would change, when the sequence of the pools is changed (Figure 4). However, a model with an appropriate age-dependency estimated the parameter values correctly even when the sequence of the pools was switched, but this will inevitably affect the estimated digestibility as discussed later.

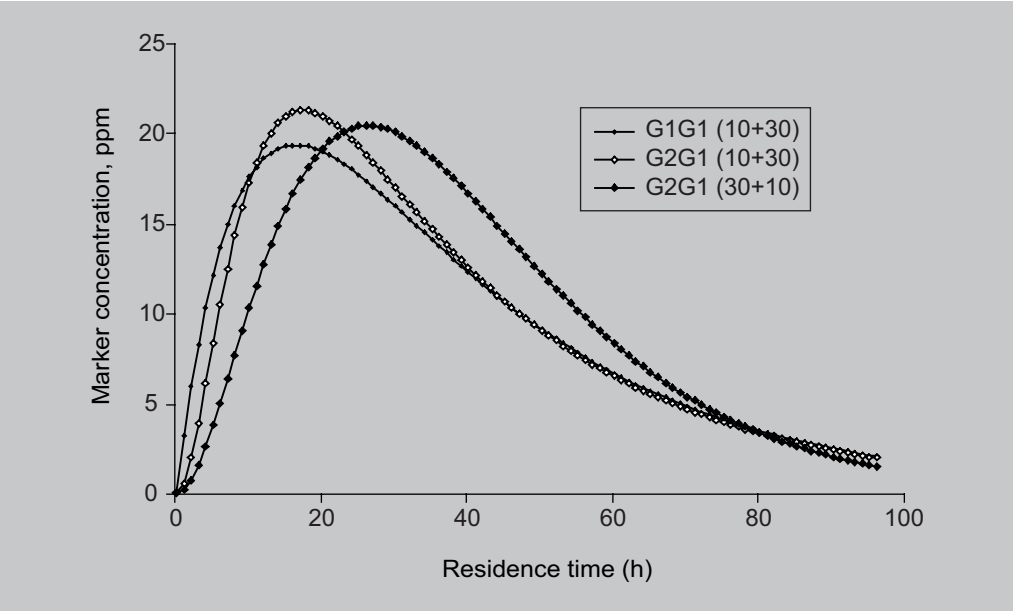

Figure 4. Simulated duodenal marker concentrations when the residence time in the two rumen compartments are 10 + 30 h or 30 + 10 h with model of no time dependency (G_1G_1) or gamma two time dependency in the first compartment (G_2G_1). Note that the two curves with the G_1G_1 model are exactly similar and only 10 + 30 h is shown.

Intrinsic and extrinsic factors influencing passage kinetics

The importance of passage rate on intake and digestibility was clearly outlined by Blaxter *et al.* (1956) and Waldo *et al.* (1972). It is often difficult to conclude whether intrinsic characteristics of particles or diet type have a greater influence on passage kinetics. Vega and Poppi (1997) addressed this question by labelling small (0.5-1.2 mm) grass and legume particles which had either been extensively digested (faecal particles) or not digested (ground feed particles) and inserted the particles into the rumen of sheep fed four different diets. The passage rate of particles was similar within a diet, irrespective of the type (grass vs. legume) or status (undigested vs. digested) of particles. Rumen conditions affected by diet type had the most influence on particle passage rate. It is therefore possible that the passage kinetic parameters are often a reflection of the effects of feed intake rather than the intrinsic plant characteristics. Welch (1982) assessed the effects of diet and feeding level on rumen raft consistency by measuring the rate of vertical penetration through the raft under a constant force. The rumen raft was more tightly packed with steers offered grass hay compared with maize silage or high levels of concentrate. Increases in feeding level from 0.40 to 1.0 of *ad libitum* intake increased rumen raft consistency.

Accurate passage kinetics data are a prerequisite for any nutritional model which attempts to predict the relationship between diet and nutrient supply. Many empirical models have been developed to predict passage kinetic parameters from dietary and animal data (e.g. Owens and

Goetch, 1986; Sniffen *et al*., 1992; Cannas *et al*., 2003). However, the predictions have not been very accurate, which may, at least in part, be explained by aspects of the methodologies used. In addition to the true intrinsic and extrinsic factors involved, factors such as marker type, kinetic model, sampling site and physical form of the marker can markedly influence the passage kinetic values obtained. To predict the actual passage kinetics it is important that the labelled particles simulate the passage of natural feed components. Without an accurate description of the particle size distribution of markers it is difficult to interpret the passage kinetics parameters from different studies. As an example, increasing the particle size of mordanted hay from <0.3 mm to 0.6-1.0 mm decreased passage rate from 0.041 to 0.021 h^{-1} (Bruining and Bosch, 1992). Both values may describe relative differences due to diet and animal on passage kinetics, but not necessarily indicate the intrinsic kinetic properties of natural feeds.

The wide range of methods and procedures that have been used to quantify particle kinetics and the biological mechanisms have been reviewed by Kennedy and Murphy (1988) and Kennedy and Doyle (1993). It was postulated that the intrinsic characteristics of plant cell walls influencing passage kinetics are mainly associated with the resistance of cell walls to comminution and particle-size reduction.

Although the resistance to comminution might be expected to be lower for early harvested forages, there is evidence that rumen retention time decreases with increasing cell wall content and maturity. Gasa *et al*. (1991), Bosch *et al*. (1992) and Bosch and Bruining (1995) observed that late cut grass silage with a higher NDF concentration resulted in a faster passage rate of Cr-mordanted fibre than grass silage harvested earlier. Rinne *et al*. (2002) found using rumen evacuation technique a significantly increased passage rate with the most delayed harvest time in dairy cows offered diets based on grass silage harvested at different stages of maturity. The proportion of large particles (>2.5 mm) of rumen particulate DM (particles >0.08 mm) decreased from 0.56 for the earliest cut to 0.43 with the latest cut. The slower breakdown of large particles in the early compared with late harvested silage suggests either a preferential removal of stems from the large-particle pool to the small-particle pool, or that the fragility of both leaves and stems increase with advancing maturity. Whether the increase in passage rate with advancing maturity of grass is an intrinsic property or a reflection of differences in intake, remains unclear. In the study of Rinne *et al*. (2002), DM intake decreased with greater maturity, but NDF intake increased due to the proportionally higher increase in silage NDF concentration which more than compensated for the reduction in DM intake. Similarly, Lund (2002) reported a faster passage rate of INDF with increased maturity using the rumen evacuation method when comparing silages harvested at 3 week intervals when forage was the sole feed, but this was not the case when the diets were supplemented with concentrate. In contrast to the studies referred above, Ellis *et al*. (2000) found that CMRT increased with increasing NDF concentration, but they did not define the details of the forages fed. However, based on INDF concentration, these forages were of lower quality than grass silage.

Kuoppala *et al*. (2004) compared the passage kinetics of primary and re-growth grass silages each harvested at two maturities using the rumen evacuation technique in dairy cows. Within harvests, the passage rate of INDF was not influenced by maturity, but it was markedly slower for the re-growth than primary growth silages (0.027 vs. 0.021 h^{-1}) despite the higher proportion of leaves

in the re-growth grass. Because the intake of cell wall and DM were higher for primary-growth silages, no definite conclusions on the cause and effect can be made: was the lower intake of re-growth silages mediated by metabolic constraints that reduced passage rate or did the intrinsically slower passage rate of re-growth grass constrain intake. A slower passage rate of silage with a higher leaf proportion is in contrast with the observations of Poppi *et al*. (1981) and Cherney *et al*. (1991), who reported that retention time was shorter for leaves than stems for various forage species. More research is needed to assess the relative importance of the intrinsic properties of forages (e.g. potential NDF digestibility, leaf to stem ratio, legume *vs*. grass, particle size) and animal/diet factors in the regulation of passage kinetics.

Comparison of the passage kinetics of markers dosed either as large or small (ground feed or faeces) particles have clearly shown differences in total retention time (Cherney *et al*., 1991; Mambrini and Peyrand, 1997; Wylie *et al*., 2000; Ahvenjärvi *et al*., 2004). The differences in total mean retention time have been approximately 10 h. However, very little is known about the effects of forage harvesting techniques on passage kinetics. Differences in the particle size of forages are distinctly smaller than differences in the particle size of markers used in these studies. It is possible that within the range of chop lengths on-farm the effect on intrinsic passage kinetic parameters is likely to be small. However, as demonstrated by Vega and Poppi (1997), the diet fed to animals often has a much stronger effect on passage rate than the properties of the labelled feed itself. It could be speculated that with fine chopping of forages the ability of the mat to entrap small particles may be reduced resulting in lowered residence time in the rumen. Bernard *et al*. (2000) replaced chopped orchard-grass with ground and pelleted orchard-grass. Grinding and pelleting of hay clearly decreased the mean residence time of lignin in the rumen when the proportion of ground hay was 0.50. No further decreases were observed at higher inclusion rates of ground pelleted hay. Shaver *et al*. (1986) fed pre-bloom lucerne hay in long, chopped or ground and pelleted form (60:40 forage to concentrate ratio DM basis) to dairy cows at three stages of lactation. Total mean residence time of labelled concentrates and forages decreased with increasing feed intake, but there were no effects of chopping or grinding on ruminal or total residence time. An absence of a difference between the long and chopped (mean particle length 7.8 mm) hay indicates that within the normal range achieved in practice, the effects of chop length on digesta passage kinetics is insignificant. Depression of digestibility associated with grinding was related to reduced ruminal digestion rate. In published studies the effects of grinding on rumen or total retention time have been variable (see Bernard *et al*., 2000). Effects of forage conservation methods on passage kinetics are likely to be small. Udén (1984) reported similar values using Cr-mordanted fibre for the passage kinetic parameters of the cows fed silage or hay. Huhtanen and Jaakkola (1993) used the rumen evacuation technique to study passage kinetics of grass silage and hay made from the same sward. The differences between the forages, although sometimes significant, were relatively small. Particle passage kinetics of lucerne hay and silage measured using ^{15}N enrichment of acid detergent fibre bound nitrogen as an internal marker were found to be similar (Huhtanen and Hristov, 2001).

Rumen residence time of concentrate particles is shorter than that of forages (e.g. Shaver *et al*., 1988; Colucci *et al*., 1990) reflecting the smaller particle size and higher specific gravity. Offer and Dixon (2000) compiled data in the literature and concluded that the effects of supplement composition on passage rates appear to be small. Robinson *et al*. (1987) observed decreased

passage rates using both Cr-straw and rumen evacuation techniques, when the starch content in concentrates increased. However, these effects may be more related to the extrinsic effects of the diet on passage kinetics, since concentrates were not labelled. Stensig *et al*. (1998) also found that supplementation of a starch rich low fibre concentrate decreased passage rate. Huhtanen *et al*. (1993) compared the passage kinetics of Yb-labelled barley, barley fibre, rapeseed meal and soybean meal in cattle. Despite the large differences in chemical composition of the feeds with respect to starch, NDF and protein content, compartmental residence times, estimated from duodenal or faecal marker profiles, were similar. Duodenal marker profiles of labelled concentrate feeds have clearly shown an ascending phase in the marker excretion curve (Huhtanen *et al*., 1993; Mambrini and Peyraud, 1997) indicating that the passage kinetics can not be described by a first-order single pool model. The diurnal pattern of duodenal amino acid (Robinson *et al*., 2002) and starch (Tothi *et al*., 2003) flow are consistent with marker kinetic data, and clearly indicate that the passage kinetics of solid feed components cannot be described using a single compartment first-order model. Excluding the ascending phase of the marker excretion curve will markedly underestimate the retention time of concentrates in the rumen fermentation compartments.

Most of the studies have shown a decrease in CMRT with increased feed intake. Intake is used in many feed evaluation systems (e.g. AFRC, 1993; NRC, 2001, Sniffen *et al*., 1992) to predict passage rate. These relationships are based mainly on marker kinetic data. We estimated the relationship between intake and diet parameters from Danish and Finnish dairy cow studies. The data included 41 treatment means with a wide range of diets (DM intake 8.2 – 23.7 kg d^{-1}, NDF concentration 238 – 638 g kg^{-1} DM, proportion of concentrate 0.00 – 0.70). The passage rate of INDF was estimated using the rumen evacuation technique, i.e. it describes the passage rate of the total diet. Intake, rather than faecal output of INDF was used to estimate the passage rate. When analysed with a single regression model, NDF intake predicted INDF passage rate much better than DM intake (R^2 0.68 vs. 0.31). Accounting for the random effect of study in a mixed model regression analysis did not change the parameter values, but the model did account for more of the observed variation (Figure 5). When NDF intake was segregated to forage and concentrate NDF the variation explained increased to 0.73 with the single regression model but was not further improved with the mixed model. The slope of the INDF passage rate was significantly higher for NDF from concentrates than forages (mixed model: 0.00034 vs. 0.00023 per kg NDF intake). This indicates that the passage rate of concentrate INDF was faster than that of forage INDF, which is consistent with the data from studies comparing the passage kinetics of labelled forages and concentrates (Shaver *et al*., 1986; Colucci *et al*., 1990; Mambrini and Peyraud, 1997). It seems that the passage rate of INDF for diets based on maize or lucerne silage is much higher compared with diets based on grass silage in relation to NDF intake. The mean NDF intake and INDF passage rate for 8 diets in the studies of Oba and Allen (2003) and Voelker and Allen (2003) were 5.7 kg d^{-1} and 0.035 h^{-1}, respectively. The passage rate of INDF was markedly higher than a value of 0.020 h^{-1} predicted by the equation derived from our dataset. Lund (2002) also found a markedly higher INDF passage rate for maize silage compared with other forages. There may be differences in the consistency of the rumen raft due to intrinsic differences between the forages that explain this finding. Bruining *et al*. (1998) estimated using a steady-state procedure that comminution rates for diets based on maize or lucerne silages (0.043 and 0.049 h^{-1}) were clearly higher than for a diet based on grass (0.034 h^{-1}).

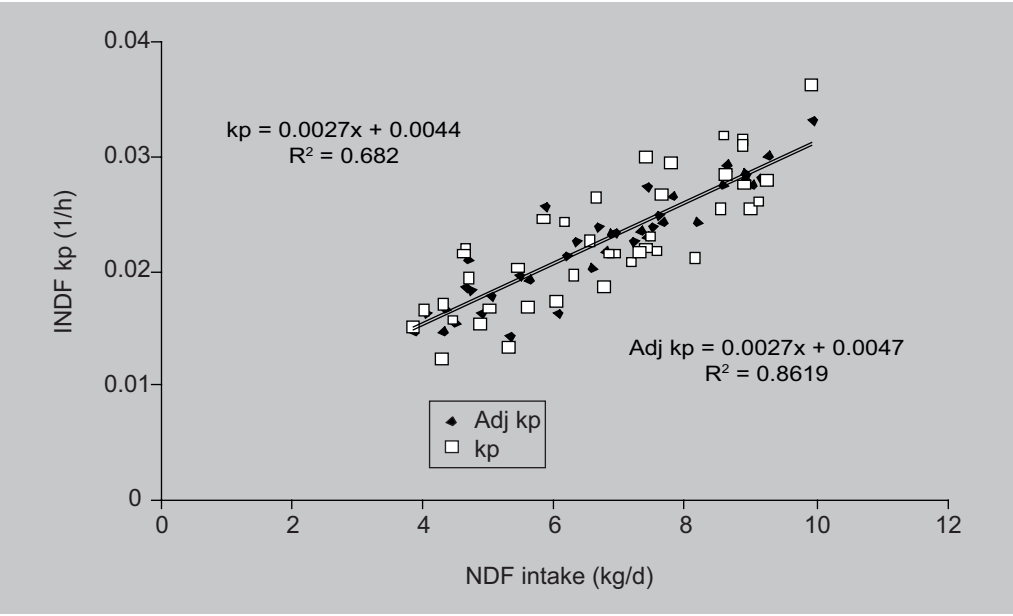

Figure 5. The relationship between NDF intake and passage rate of INDF estimated by rumen evacuation technique. The data from the Danish and Finnish studies was analysed either by single regression or by a mixed model with random study effect. Adj kp: Values are adjusted for a random study effect.

The relationship between NDF intake and INDF passage rate in our data was linear. Cannas *et al*. (2003) observed that the relationship between lignin turnover and NDF intake was best described by a concave curve (NDF intake was transformed by the natural logarithm). However, because the inverse of passage rate represents turnover, the relationship between NDF intake and NDF rumen turnover time are consistent by Cannas *et al*. (2003). Cannas and Van Soest (2000) showed that forage NDF passage rate, estimated by external markers, was best described by a convex curve, i.e. at high levels of intake passage rate increased to a lower extent than at low levels of intake. If this relationship were true, the rumen NDF pool would increase exponentially with feed intake unless the rate of digestion increased, but this does not appear to be the case (Robinson *et al*., 1987). Tamminga *et al*. (1989) estimated passage rate using both Cr-mordanted straw as an external marker and the rumen evacuation technique in dairy cows fed at different levels of intake. Passage rate of INDF increased linearly (or even slightly curvilinearly) with increased intake, whereas the pattern was not so clear with Cr-mordanted straw. It is possible that the relationships between intake and passage rate in this experiment were influenced by the problems related to marker kinetics data, the most serious problem being that the passage rate estimated from marker profiles did not account for the residence time represented by the ascending phase of the marker excretion curve, and the inverse of marker passage rate therefore underestimates rumen turnover.

Reducing effects of increased concentrate in the diet on passage rate were reported by Colucci *et al*. (1990), Gasa *et al*. (1990), Bosch *et al*. (1992) and Huhtanen and Jaakkola (1993). In the study

of Colucci *et al.* (1990) increasing the proportion of concentrate showed a significant negative relationship with passage at a low level of feeding, but at a higher level of feeding the effects were small and non-significant. Huhtanen and Jaakkola (1993) used the rumen evacuation technique to estimate passage kinetics of cell walls in cattle fed at a fixed DM intake of diets supplemented with three levels of concentrate. Increasing the level of concentrate in the diet decreased the passage rate of INDF linearly, even though the proportion of concentrate NDF of intrinsically higher flow characteristics increased.

The rumen evacuation technique appears to be a useful tool for estimating passage kinetics of INDF. More detailed analysis of larger sets of data might be useful for the estimation the effects of diet, animal and feed characteristics on INDF passage kinetics. The problem with this method relates to the fact that it estimates the passage rate for the total diet and not for individual ingredients. A combination of marker and rumen evacuation techniques may be useful to separate the effects due to intrinsic and extrinsic factors on passage kinetics. For the estimation of intrinsic feed characteristics, the labelled feed should be fed in the same physical from as it is fed in the diet. Extrinsic effects on passage kinetics can be estimated by using one common marker for all diets in a study or by the rumen evacuation technique.

Integrated models of cell wall digestion in the rumen

Random passage models

The original model with random passage of Waldo *et al.* (1972) has been the basis of dynamic rumen models predicting cell wall digestibility. The model involved a concept of potential digestibility, fractional rates of digestion and passage and that digestibility is a competition between digestion and passage. The simple model has been modified, but most of the published data on predicted NDF digestibility estimated from the kinetic parameters are still based on this concept. The use of this model has been extended to estimate the effective protein degradability in the rumen (Ørskov and McDonald, 1979), and is probably more widely used for that purpose than predicting NDF digestibility.

Integrated models of cell wall digestion have been extensively reviewed (Allen and Mertens, 1988; Mertens, 1993a; Illius and Allen, 1994; Ellis *et al.*, 1994). The models have considerable differences in substrate fractionation and in the structures applied to describe digestion and passage kinetics. The abilities of the published models to predict digestibility have not been particularly reassuring (Illius and Allen, 1994). To be useful for practical feed evaluation and ration formulation purposes, integrated models should predict digestibility at least with the same accuracy as models based on empirical relationships between digestibility and selected chemical components. Our objective here is to discuss the effects of model structure on the prediction of NDF digestibility and the possible mechanisms and processes that relate to the model structure that should be used.

Although the original model of Waldo *et al.* (1972) has been widely used to calculate NDF digestibility in many studies, it has not been extensively validated against *in vivo* data based on a large number of measurements. Archimède (1992) used *in situ* digestion kinetic data to predict

ruminal NDF digestibility estimated from duodenal flow (cited by Nozière and Michalet-Doreau 2000). The model clearly underestimated ruminal NDF digestibility, but the slope between predicted and observed values was 1.00 and the proportion of variance accounted for by this model was relatively high ($R^2 = 0.65$). Underestimation of *in vivo* digestibility was suggested to be due to the underestimation of digestion rate by the *in situ* technique. This model probably results in the correct ranking of NDF digestibility, since the empirical relationship between INDF concentration and in *vivo* OM digestibility is particularly strong (Nousiainen *et al*., 2003b). In addition to the underestimation of the rate of digestion, too high passage rates from ignoring the ascending phase of the marker excretion curve can lead to an underestimation of NDF digestibility with this model.

Knowledge of *in vivo* digestibility of DNDF can be used to validate the feasibility of a model structure. The mean DNDF digestibility of 52 grass silages harvested at different stages of maturity was 0.87 (range 0.79 – 0.93) (Nousiainen *et al*., 2004), such that using a passage rate of 0.02 h^{-1}, indicates that digestion rate should be 0.075, 0.128 and 0.260 h^{-1} to achieve the observed minimum, mean and maximum *in vivo* DNDF digestibility. Accordingly, using a digestion rate of 0.06 h^{-1}, a passage rate of 0.0094 h^{-1} would be required to achieve the observed mean *in vivo* DNDF digestibility. These simple calculations indicate that unrealistic values for the digestion and/or passage rates have to be used in order to predict *in vivo* DNDF digestibility correctly, when inappropriate models are used. It has sometimes been argued that the hind-gut digestion compensates for the difference between predicted and observed digestibility (Moore *et al*., 1990). However, in animals fed forage diets the contribution of the hind-gut to total NDF digestion cannot be that large as discussed earlier in this chapter. Another explanation for the underestimation of NDF digestibility is that passage rates used in various models are often estimated from the descending phase of marker excretion curves. It can be concluded that although the model has many basic elements of the dynamics of cell wall digestion, cell wall digestion can not be correctly estimated by a simple mathematical function based on random passage and the model is not biologically sound either.

Selective retention models

Ruminants have evolved an effective system of selective retention to maximise the intake of digestible energy by retaining the newly ingested digestible and large particles in the rumen and allowing the passage of aged particles, which are small and depleted of digestible material. Although the mechanisms of selective retention are well described in the literature (Kennedy and Murphy, 1988; Sutherland, 1988; Allen and Mertens, 1988; Kennedy and Doyle, 1993) and have been incorporated in many integrated rumen models (Illius and Allen, 1994), it has not been integrated in feed evaluation systems and very seldom in the calculation of NDF digestibility from kinetic data (e.g. Sniffen *et al*., 1992; AFRC, 1993; NRC, 2001).

A two-compartment model incorporating indigestible and digestible NDF is illustrated in Figure 6. From the first compartment (lag-rumination pool) DNDF disappears by digestion and comminution to the second compartment (small particle pool). From the second compartment DNDF disappears both by digestion and passage. By definition INDF disappears from the first compartment only by release to the second compartment and from that by passage to the small

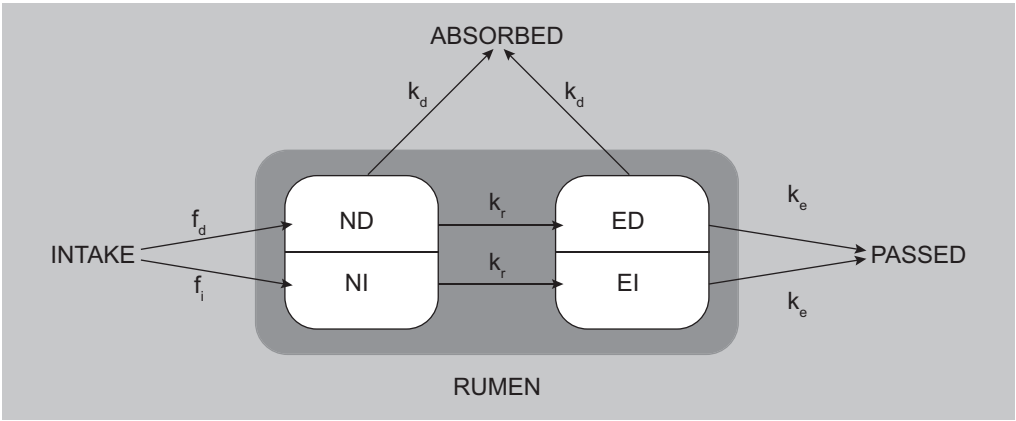

Figure 6. Model of ruminal cell wall digestion incorporating selective retention of potentially digestible (D) and indigestible (I) fractions in non-escapable (N) and escapable (E) pools. Cell wall fractions and rates are represented as follows: digestible NDF (f_d), indigestible NDF (f_i), rate of digestion (k_d), rate of release from N to E (k_r) and rate of escape from E (k_e) (Allen and Mertens, 1988).

intestine. Derivation of digestibility was presented by Allen and Mertens (1988) (equation 4 in this chapter). The use of this model results in much more realistic values of *in vivo* DNDF digestibility than the single compartment model.

An example of the effects of selective retention and time dependency of the flow in the first compartment (Ellis *et al.*, 1994) on ruminal DNDF digestibility is shown in Figure 7. Inclusion of the selective retention of particles in the model increased ruminal DNDF digestibility from 0.69 to

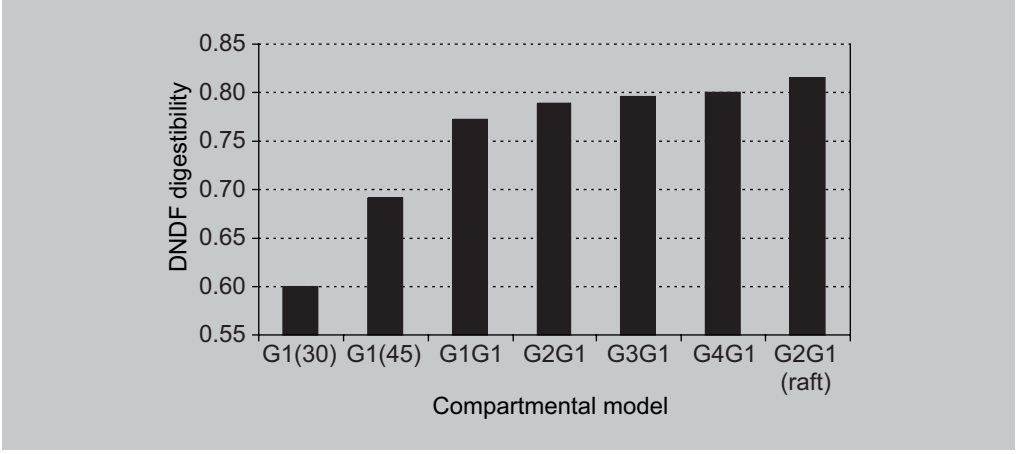

Figure 7. The effects of compartmental passage model on estimated DNDF digestibility. One compartment models (G1) assume either the same compartmental residence time (45 h) or that it was estimated from the ascending phase of the marker excretion curve (30 h). A value of 0.05 h^{-1} was used for the rate of digestion.

0.77 when the same rumen residence time of 45 h (15+30) was used. Assuming an age-dependent flow from the first to the second compartment resulted in a further 0.02-0.03 unit improvement in DNDF digestibility. If the passage rate obtained from the slower compartment of the two compartment model (30 h) had been used in a single compartmental model, estimated DNDF digestibility would have been only 0.60. However, this is the approach used in most published data and also in feed evaluation systems.

In a single compartment system with first-order digestion and passage rates, the proportions of DNDF disappearing via digestion per definition remain constant throughout a range of residence times. In a two compartment system the proportion of DNFD disappearing by digestion is much higher during earlier residence times when the particles are mainly in the lag-rumination pool and not eligible for passage (Figure 8). The proportion of DNDF disappearing by passage in the two compartment system is smaller during the early residence time compared with a single compartmental system, but for later residence times the reverse is true. Due to the slow disappearance via passage during early residence times a digestion lag time would have a smaller effect than in a single compartmental system (Ellis *et al.*, 1994). Allen and Mertens (1988) showed mathematically that if the lag phenomena influence both digestion and passage, cell wall digestibility is independent of lag. They also derived an equation to calculate simple first-order passage rate from the three kinetic parameters:

$$k_p = k_r \text{ x } k_e / (k_{d+} k_r + k_e) \quad (6)$$

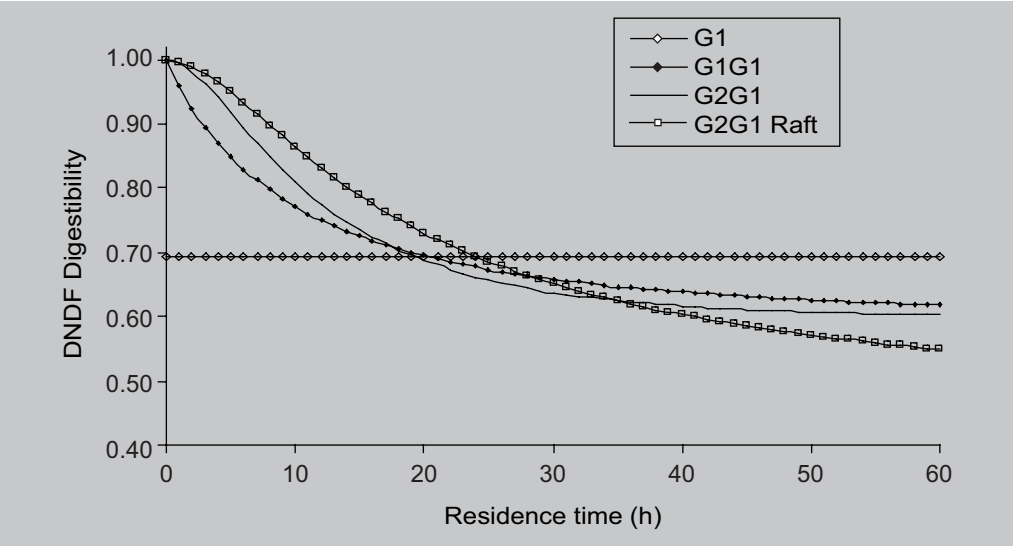

Figure 8. Digestibility of DNDF (proportion of DNDF disappearing by digestion) at different rumen residence times assuming rumen as a single compartment (G_1) or two compartment system. In the two compartment systems the flow followed first-order kinetics (G_1G_1) or the flow from the first compartment was age-dependent (G_2G_1). The distribution of the residence time was 15 + 30 h for the G_1G_1 and G_2G_1 systems and 30 + 15 h for the G_2G_1 raft system. Digestion rate was assumed to be 0.05 h^{-1}.

where k_d, is the rate of digestion, k_r is release from the non-escapable to the escapable compartment and k_e is passage from the escapable compartment to the lower tract. The first-order passage rate is not only a function of the passage kinetic parameters but also of the fractional digestion rate. At a constant CMRT, the higher the rate of digestion, the lower the first-order passage rate would be. It might be argued that estimating parameter values of the model is difficult. However, for the determination of digestibility, an accurate estimate of total residence time in the two compartments is much more important than the distribution of the residence time between the two compartments. If the distribution of the total residence time of 45 h were 10+35, 15+30 or 20+25, the calculated DNDF digestibility would be 0.758, 0.771 and 0.778, respectively. This example suggests that digestibility is relatively insensitive to small changes in the distribution of the residence time between the two compartments. However, with large changes the model will approach a single compartment model and have a large impact. The raft model recently suggested by Poppi *et al.* (2001) would result in a distinctly higher DNDF digestibility provided that passage from the raft pool to the passage pool is a time dependent process, as their data suggest. When the same digestion rate (0.05 h^{-1}) and total compartmental retention time (45 h) were used in the model but assuming a gamma two time dependency in the raft pool with a distribution of 35+10 h in the two compartments, DNDF digestibility was predicted to be 0.811 (see Figure 7). This suggest that the raft model is more effective in maximising the efficiency of ruminal DNDF digestion than models assuming a shorter retention time in the first than second compartment. More efficient digestion is related to the greater proportion of DNDF disappearing by digestion during early residence times when the particles become more slowly available for escape (See Figure 8). The data of Ahvenjärvi *et al.* (2001) support the concept of a raft model. In this study, cows were fed grass silage as the sole feed and digestibility of DNDF was shown to be very high (0.89) despite the relatively high NDF intake (12 g/kg LW). The similar particle size distribution and potential NDF digestibility within each particle size fraction in the rumen ventral and dorsal sacs are suggestive of a raft model concept rather than of selective retention based on particle flotation and sedimentation.

The validity of the two compartment model has not been extensively tested against *in vivo* data. Ellis *et al.* (1994) discussed that without the mechanisms of selective retention in the rumen it would be impossible to attain observed *in vivo* DNDF digestibility with realistic parameter values. Mertens (1973) concluded that assuming the rumen as a single compartment is not an adequate mathematical or biological representation of rumen functions. Huhtanen *et al.* (1995) and Rinne *et al.* (1997) predicted *in vivo* NDF digestibility using digestion rates derived from *in situ* incubations or from rumen evacuation and passage kinetic parameters estimated from the marker profiles. The mean NDF digestibility predicted using a two compartmental system and digestion rates based on rumen evacuation or in situ were 0.725 and 0.581, respectively, but only 0.424 when estimates were based *on situ* digestion rate and passage rate assuming the rumen to be a single compartment. All systems ranked the diets correctly, but the latter models clearly underestimated NDF digestibility (0.728). The data suggests two obvious reasons for the underestimation of digestion: first, the *in situ* method underestimates the rate of digestion and second, the assumptions of a single rumen compartment were not correct. Huhtanen *et al.* (2001) estimated NDF digestibility of 15 grass silages in sheep fed at maintenance with a two compartment rumen model assuming a total residence time 50 h (20+30 h). Digestion rate was estimated by the gas production technique from isolated NDF and potential NDF digestibility by

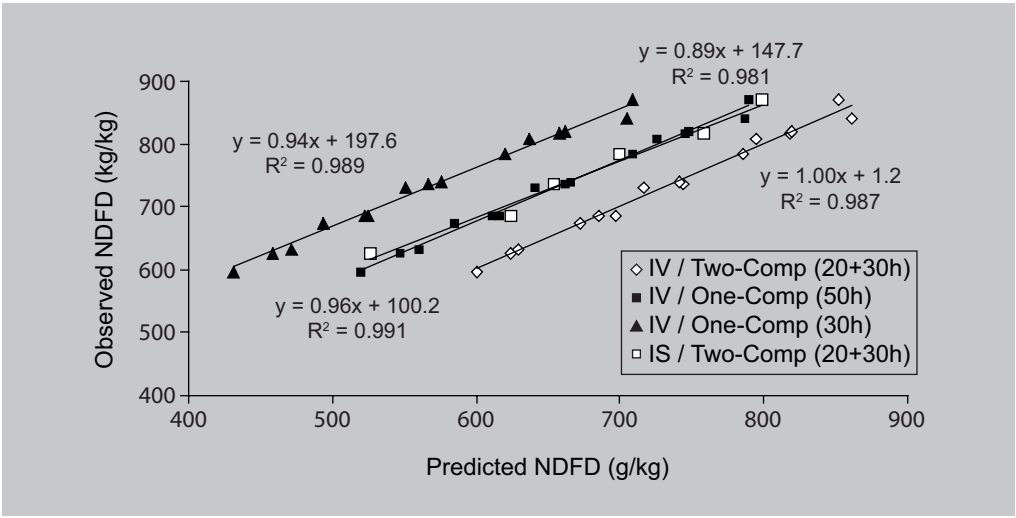

Figure 9. The relationship between predicted and observed NDF digestibility (NDFD) in sheep fed at maintenance. NDFD values were predicted using digestion rates determined by in vitro gas production (IV) or by in situ incubation (IS) using a two compartment rumen model (residence time 20+30h) or one compartment rumen model (residence time 50 or 30 h). (Data from Huhtanen et al.*, 2001 and unpublished data).*

12 d *in situ* incubations. The model predicted NDF digestibility accurately without any mean or slope bias (Figure 9). Using a single compartmental model with the same residence time clearly underestimated NDF digestibility, but did not change the proportion of variance accounted for by the model. *In situ* rate of digestion was determined for six of the 15 silages. Again, the method ranked the feeds correctly, but the lower predicted NDF digestibility suggests that the *in situ* method underestimated the rate of digestion.

The basal model structure in the Nordic dairy cow model (Danfær *et al.*, 2005a) is a two compartment rumen system and a single hind-gut compartment. Cell walls are fractioned into digestible and indigestible forage and concentrate NDF. Digestion of cell walls is assumed to be a first-order process. Intrinsic ruminal DNDF digestion is regulated by the ratio of non-structural carbohydrates and NDF, which models the adverse effects of rapidly degradable carbohydrates on cell wall digestion. Cell wall digestion is assumed to take place both in the rumen and in the hind-gut. Passage rate is regulated by feed intake in terms of NDF per unit live weight. Higher passage rates are used for concentrates than for forages. The proportion of the lag-rumination (non-escapable) compartment of the total rumen residence time is assumed to be 0.30 and 0.20 for forages and concentrates, respectively. Preliminary validation of the model indicates that this approach provides an accurate prediction of ruminal and total NDF digestibility with minimal mean and slope bias (Danfær *et al.*, 2005b). The relationship was much stronger when the values were adjusted for the random effects of experiment (Figure 10). This indicates that the model predicted the differences between the diets within experiment very accurately, and that a large proportion of the variance in the simple regression analysis arises from methodological

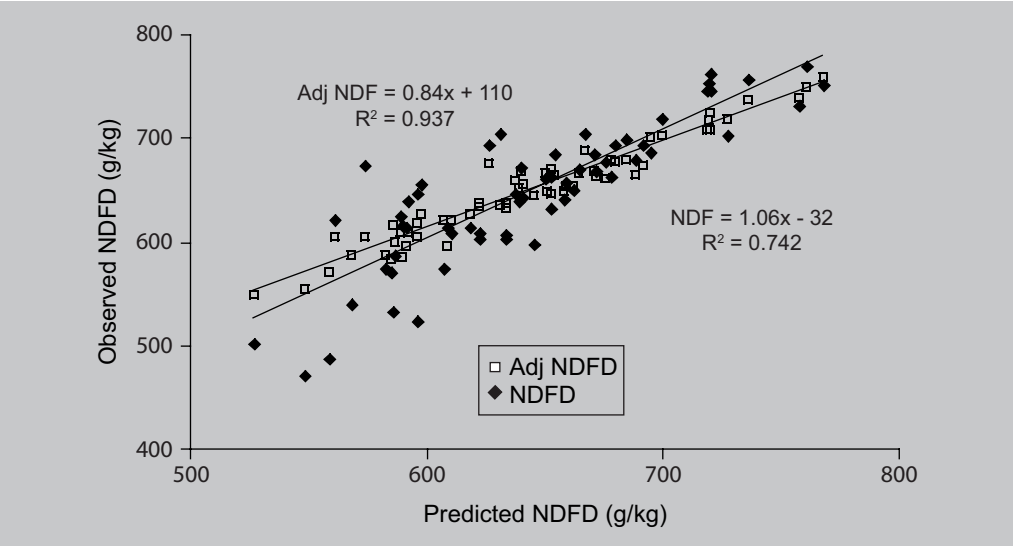

Figure 10. Relationship predicted (Nordic Dairy Cow Model; Danfær et al.*, 2005a) and observed NDF digestibility (NDFD) with or without adjustments for random experimental effects.*

differences in the determination of digestion kinetic parameters. The close relationship between digestion rates determined by different methods support this suggestion.

Conclusions

Development of useful mechanistic models for estimating digestibility and intake require both an accurate estimation of the parameter values and appropriate model structure. More work is required to validate the methods used to estimate digestion kinetic parameters. Most of the validation has been conducted by comparing two systems (e.g. *in vitro* vs. *in situ*) without validation against reliable *in vivo* data. Most of the systems appear to rank the feeds reasonably well, but that is not a satisfactory criterion to be of use for mechanistic rumen models. It appears that none of the present methods fulfil the requirements of an ideal method. It is important that only the intrinsic characteristics of cell walls limit the rate and extent of digestion, i.e. that the system itself is not a limiting factor. Future work is also required to estimate quantitative effects of some extrinsic factors such as intake and diet composition on the intrinsic rate of cell wall digestion.

A better understanding of the effects of, and interactions between, intrinsic and extrinsic factors on passage kinetics are required. In many cases estimated passage kinetic parameters represent interactions between animal and feed characteristics. In the future, more attention should be paid to distinguishing between animal (e.g. intake, diet composition) and intrinsic feed characteristics. Also more validation of the current marker systems against preferred reference methods (slaughter, rumen evacuation, appropriate internal markers) needs to be conducted.

Even though the mechanisms of selective retention of feed particles have been unequivocally described, it has seldom been used to calculate NDF digestibility from kinetic parameters. This fundamental flaw in the model structure, used extensively in most feed evaluation systems, leads to serious underestimations of NDF digestibility. When the mechanisms of selective retention of feed particles in the mechanistic rumen models are ignored, unrealistically high digestion rates and/or low passage rates have to be used to correctly predict *in vivo* digestibility. For an accurate prediction of NDF, and consequently OM digestibility, reliable estimates of intrinsic digestion kinetics and an adequate description of the underlying digestion and passage processes are required, but it is also essential that the regulation of intrinsic digestion rate and rumen residence time due to diet composition and level of intake are taken into account. For further progress in developing useful mechanistic models for the prediction of digestibility and intake, it is vital that modellers and ruminant biologists work in harmony.

References

AFRC (Agricultural and Food Research Council) (1993). Energy and protein requirements of ruminants. CAB International, Wallingford, UK, 183pp.

Ahvenjärvi S., A. Vanhatalo, P. Huhtanen & T. Varvikko (1999). Effects of supplementation of a grass silage and barley diet with urea, rapeseed meal and heat-moisture-treated rapeseed cake on omasal digesta flow and milk production in lactating dairy cows. Acta Agriculturae Scandinavica, Section A, Animal Science, 49, 179-189.

Ahvenjärvi, S., B. Skiba & P. Huhtanen (2001). Effect of heterogeneous digesta chemical composition on the accuracy of measurements of fiber flow in dairy cows. Journal of Animal Science, 79, 1611-1620.

Ahvenjärvi, S., A. Vanhatalo, A.N. Hristov & P. Huhtanen (2004). Passage kinetics of internal and external markers in lactating dairy cows. Journal of Animal and Feed Sciences 13, Supplement 1, 19-22.

Aitchison, E., M. Gill, J. France & M.S. Dhanoa (1986). Comparison of methods to describe the kinetics of digestion and passage of fibre in sheep. Journal of the Science of Food and Agriculture, 37, 1065-1072.

Allen, M.S. & D.R. Mertens (1988). Evaluation constraints of fibre digestion by rumen microbes. Journal of Nutrition, 118, 261-270.

Archimède, H. (1992). Etude des facteurs impliqués dans les interactions digestives entre les fourrages et les aliments concentrés chez les ruminants. Thèse de doctorat, INRA-PG, 144pp.

Bernard, L., J.P. Chaise, R. Baumont & C. Poncet (2000). The effect of physical form of orchardgrass hay on the passage of particulate matter through the rumen of sheep. Journal of Animal Science, 78, 1338-1354.

Beauchemin, K.A. & J.G. Buchanan-Smith (1989). Evaluation of markers, sampling sites and models for estimating rates of passage of silage and hay in dairy cows. Animal Feed Science and Technology, 27, 59-75.

Blaxter, K.L., N. McC. Graham & F.W. Wainman (1956). Some observations on the digestibility of food by sheep and on related problems. British Journal of Nutrition 10, 69-91.

Blümmel, M. & E.R. Ørskov (1993). Comparison of *in vitro* gas production and nylon bag degradability of roughages in predicting feed intake in cattle. Animal Feed Science and Technology, 40, 109-119.

Bosch, M.W. & M. Bruining (1995). Passage rate and total clearance rate from the rumen of cows fed grass silage differing in cell wall content. British Journal of Nutrition, 73, 41-49.

Bosch, M.W., S.C.W. Lammers-Weinhoven, G.A. Bangma, H. Boer & P.W.M. van Andrichem (1992). Influence of the stage of maturity of grass silages on digestion processes in dairy cows. 2. Rumen contents, passage rates, distribution of rumen and faecal particles and mastication activity. Livestock Production Science, 32, 265-281.

Bowman, J.G.P., C.W. Hunt, M.S. Kerley & J.A. Paterson (1991). Effect of grass maturity and legume substitution on large particle size reduction and small particle flow from the rumen of cattle. Journal of Animal Science, 69, 369-378.

Bruining, M., R. Bakker, J. van Bruchem & S. Tamminga (1998). Rumen digesta kinetics in dairy cows fed grass, maize and alfalfa silage. 1. Comparison of conventional, steady-state and dynamic methods to estimate microbial degradation, comminution and passage of particles. Animal Feed Science and Technology, 73, 37-58.

Bruining, M. & M.W. Bosch (1992). Ruminal passage rates affected by CrNDF particle size. Animal Feed Science and Technology, 37, 193-200.

Cannas, A. & P.J. Van Soest (2000). Simple allometric models to predict rumen feed passage rate in domestic ruminants. In: J.P. McNamara, J. France & D.E. Beever (eds.) Modelling Nutrient Utilization in Farm Animals. CAB International, Wallingford, UK, pp 49-62.

Cannas, A., P.J. Van Soest & A.N. Pell (2003). Use of animal and dietary information to predict rumen turnover. Animal Feed Science and Technology, 106, 95-117.

Cherney, D.J.R., D.R. Mertens & J.A. Moore (1991). Fluid and particulate retention times in sheep as influenced by intake level and forage morphological composition. Journal of Animal Science, 69, 413-422.

Cherney, D.J.R., J. Siciliano-Jones & A.N. Pell (1993). Technical note: forage *in vitro* dry matter digestibility as influenced by fiber source in the donor cow diet. Journal of Animal Science, 71, 1335–1338

Colucci, P.E., L.E Chase & P.J. Van Soest (1982). Feed intake, apparent diet digestibility, and rate of particulate passage in dairy cattle. Journal of Dairy Science, 65, 1445-1456.

Colucci, P.E., G.K. MacLeod, W.L. Grovum, I. McMillan & D.J. Barney (1990). Digesta kinetics in sheep and cattle fed diets with different forage to concentrate ratios at high and low intakes. Journal of Dairy Science, 73, 2143-2156.

Combs, D.K., R.D. Shaver & L.D. Satter (1992). Retention of rare earths by hay particles following incubation in fresh or autoclaved rumen fluid. Journal of Dairy Science, 75, 132-139.

Cone, J.W., A.H. van Gelder & F. Driehuis (1997). Description of gas production by a three-phasic model. Animal Feed Science and Technology, 66, 31-45.

Cone, J.W., A.H. van Gelder, I.A. Soliman, A. De Visser & A.M. van Vuuren (1999). Different techniques to study rumen fermentation characteristics of maturing grass and grass silage. Journal of Dairy Science, 82, 957-966.

Cone, J.W., A.H. van Gelder & H. Valk (1998). Prediction of nylon bag degradation characteristics of grass samples with the gas production technique. Journal of the Science of Food and Agriculture 77, 421-426.

Cone, J.W., A.H. van Gelder, G.J.W. Visscher & L. Oudshoorn (1996). Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Animal Feed Science and Technology, 61, 31-45.

Danfær, A., P. Huhtanen, P. Udén, J. Sveinbjörnsson & H. Volden (2005a). Karoline - a Nordic cow model for feed evaluation - model description. In: E. Kebreab, J. Dijkstra, A. Bannink, J. Gerrits & J. France (eds.) Nutrient Didestion and Utilization in Farm Animals: Modelling approaches. CAB International, pp. 383-406.

Danfær, A., P. Huhtanen, P. Udén, J. Sveinbjörnsson, H. Volden (2005b). Karoline - a Nordic cow model for feed evaluation – model validation. In: E. Kebreab, J. Dijkstra, A. Bannink, J. Gerrits & J. France (eds.) Nutrient Didestion and Utilization in Farm Animals: Modelling approaches. CAB International, pp. 407-415.

Demeyer, D.I. & C.J. van Nevel (1995). Transformation and effects of lipids in the rumen: Three decades of research at Ghent University. Archives of Animal Nutrition, 48, 119-134.

Dhanoa, M.S., R.C. Siddons, J. France & D.L. Gale (1985). A multi-compartment models to describe marker excretion patterns in ruminant faeces. British Journal of Nutrition, 53, 663-671.

Doane, P.H., P. Schofield & A.N. Pell (1997). NDF disappearance, gas and VFA production during *in vitro* fermentation of six forages. Journal of Animal Science, 75, 3342-3352.

Doreau, M. & Y. Chilliard (1997). Digestion and metabolism of dietary fat in farm animals. British Journal of Nutrition, 78, 13-35.

Doreau, M. & A. Ferlay (1994). Digestion and utilisation of fatty acids by ruminants. Animal Feed Science and Technology, 45, 379-396.

Doreau, M. & A. Ferlay (1995). Effect of dietary lipids on nitrogen metabolism in the rumen. A review. Livestock Production Science, 43, 97-110.

Ehle, F.R., F. Bas, B. Barno, R. Martin & F. Leone (1984). Particulate rumen turnover rate measurements as influenced by density of passage marker. Journal of Dairy Science, 67, 2910-2913.

Ellis, W.C., J.H. Matis, T.M. Hill & M.R. Murphy (1994). Methodology for estimation digestion and passage kinetics of forages. In: G.C. Fahey, Jr., M. Collins, D.R. Mertens & L.E. Moser (eds.) Forage Quality, Evaluation and Utilization. American Society of Agronomy, pp. 682-756.

Ellis, W.C., D.P. Poppi, J.H. Matis, H. Lippke, T.M. Hill & F.M. Rouguette, Jr. (1999). Dietary - digestive - metabolic interactions determining the nutritive potential of ruminant diets. In: H-J.G. Jung & G.C. Fahey, Jr. (eds.) Nutritional Ecology of Herbivores. Proceedings of the Vth International Symposium on the Nutrition of Herbivores. American Society of Animal Science, pp. 423-481.

Ellis, W.C., M.J. Wylie & J.H. Matis (2002). Validity of specifically applied rare earth elements and compartmental models for estimating flux of undigested plant tissue residues through the gastrointestinal tract of ruminants, Journal of Animal Science, 80, 2753-2758.

Evans, E.W., G.R. Pearce, J. Burnett & S.L. Pillinger (1973). Changes in some physical characteristics of the digesta in the reticulo-rumen of cows fed once daily. British Journal of Nutrition, 29, 357-376.

Faichney, G.J. (1986). The kinetics of particulate matter in the rumen. In: L.P. Milligan *et al.* (eds.) Control of Digestion and Metabolism in Ruminants. Reston, Eaglewood Cliffs, New Jersey, USA, pp. 173-195.

Faichney, G.J. (1993). Digesta flow. In: J.M. Forbes & J. France (eds.) Quantitative aspects of ruminant digestion and metabolism. CAB International, Wallingford, pp. 53-85.

Firkins, J.L., M.S. Allen, B.S. Oldick & N.R. St-Pierre (1998). Modelling ruminal digestibility of carbohydrates and microbial protein flow to the duodenum. Journal of Dairy Science, 81, 3350-3369.

Firkins, J.L., M.L. Eastridge, N.R. St-Pierre & S.M. Noftsger (2001). Effects of grain variability and processing on starch utilization by lactating dairy cattle. Journal of Animal Science, 79 E. Supplement, E218-E238.

France, J., J.H.M. Thornley, M.S. Dhanoa & R.C. Siddons (1985). On the mathematics of digesta flow kinetics. Journal of Theoretical Biology, 113, 743-758.

Gasa, J., K. Holtenius, J.D. Sutton, M.S. Dhanoa & W.J. Kelly (1991). Rumen fill and digesta kinetics in lactating Friesian cows given two levels of concentrates with two types of grass silage *ad-lib*. British Journal of Nutrition, 66, 381-398.

Grant, R.J. & D.R. Mertens (1991). Effect of pH and starch on kinetics of *in vitro* fiber digestion. In: Abstracts International Symposium Forage Cell Wall Structure and Digestibility, Madison, WI. 7-10 October 1991. U.S. Dairy Forage Research Centre, Madison WI. B17pp.

Grant, R.J. & D.R. Mertens (1992). Development of buffer systems for pH control and evaluation of pH effects on fiber digestion *in vitro*. Journal of Dairy Science, 75, 1581–1587.

Grenet, E. (1989). A comparison of the digestion and reduction in particle size of lucerne hay (*Medicago sativa)* and Italian ryegrass hay (*Lolium italicum)* in the ovine digestive tract. British Journal of Nutrition, 62, 493-507.

Groleau, D. & C.W. Forsberg (1981). Cellulolytic activity of the rumen bacterium *Bacteroides succinogenes*. Canadian Journal of Microbiology, 27, 517-530.

Grovum, W.L. & V.J. Williams (1973). Passage of digesta in sheep. 4. passage of marker through the alimentary tract and the biological relevance of rate constants derived from the changes in concentration of marker in faeces. British Journal of Nutrition, 30, 313-329.

Hooper, A.P. & J.G. Welch (1985). Effects of particle size and forage composition on functional specific gravity. Journal of Dairy Science 68, 1181-1188.

Hoover, W.H. (1986). Chemical factors involved in ruminal fibre digestion. Journal of Dairy Science, 69, 2755-2766.

Hristov, A.N., S. Ahvenjärvi, T.A. McAllister & P. Huhtanen (2003). Ruminal and total digestive tract retention time of feed particles with functional specific gravity higher or lower than 1.02. Journal of Animal Science, 81, 2639-2648.

Huhtanen, P. (1987). The effects of intraruminal infusions of sucrose and xylose on the nitrogen and fibre digestion in the rumen of cattle receiving diets of grass silage and barley. Journal of Agricultural Science in Finland, 59, 405-424.

Huhtanen, P. & A.N. Hristov (2001). Estimating passage kinetics using fibre-bound ^{15}N as an internal marker. Animal Feed Science and Technology, 94, 29-41.

Huhtanen P. & S. Jaakkola (1993). The effects of forage preservation method and proportion of concentrate on digestion of cell wall carbohydrates and rumen digesta pool size in cattle. Grass and Forage Science, 48, 155-165.

Huhtanen, P. & S. Jaakkola (1994). Influence of grass maturity and diet on ruminal dry matter and neutral detergent fibre digestion kinetics. Archives of Animal Nutrition, 47, 153-167.

Huhtanen, P., S. Jaakkola & U. Kukkonen (1995). Ruminal plant cell wall digestibility and passage kinetics utilizing mathematical models. Animal Feed Science and Technology, 52, 159-173.

Huhtanen, P., S. Jaakkola, U. Kukkonen & J. Nousiainen (1993). Influence of passage model in estimating ruminal protein degradability. Journal of Animal Science, 71, Supplement 1, 263.

Huhtanen, P. & H. Khalili (1992). The effect of sucrose supplements on particle- associated carboxymethylcellulase (E.C. 3.2.1.4) and xylanase (E.C. 3.2.1.8) activities in cattle given grass-silage-based diets. British Journal of Nutrition, 67, 245-255.

Huhtanen, P. & U. Kukkonen (1995). Comparison of methods, markers and sampling sites for estimating digesta passage kinetics in cattle fed at two levels of intake. Animal Feed Science and Technology, 52, 141-158.

Huhtanen, P., M. Ots, S. Ahvenjärvi & M. Rinne (2001). Prediction of the *in vivo* digestibility of grass silage from gas production kinetics. Journal of Dairy Science, 84, Supplement 1, 197.

Huhtanen, P. & A. Vanhatalo (1997). Ruminal and total plant cell-wall digestibility estimated by a combined *in situ* method utilizing mathematical models. British Journal of Nutrition, 78, 4, 583-598.

Huhtanen, P., A. Vanhatalo & T. Varvikko (1998). Enzyme activities of rumen particles and feed samples incubated *in situ* with differing types of cloth. British Journal of Nutrition, 79, 161-168.

Hungate, R.E. (1966). The rumen and its microbes. Academic Press, New York. 533pp.

Illius, A.W. & M.S. Allen (1994). Assessing forage quality using integrated models of intake and digestion by ruminants. In: G.C. Fahey, Jr., M. Collins, D.R. Mertens & L.E. Moser (eds.) Forage Quality, Evaluation and Utilization. American Society of Agronomy, pp. 869-890.

Illius, A.W. & I.J. Gordon (1991). Prediction of intake and digestion in ruminants by a model of rumen kinetics integrating animal size and plant characteristics. Journal of Agricultural Science, Cambridge, 116, 145-157.

Kennedy, P.M. & P.T. Doyle (1993). Particle-size reduction by ruminants – Effects of cell wall composition and structure. In: H.G. Jung, D.R. Buxton, R.D. Hatfield & J. Ralph (eds.) Forage Cell Wall Structure and Digestibility. American Society of Agronomy, pp. 499-534.

Kennedy, P.M. & M.R. Murphy (1988). The nutritional implications of differential passage of particles through the alimentary tract of the ruminants. Nutrition Research Reviews, 1, 189-209.

Khalili, H. & P. Huhtanen (1991). Sucrose supplements in cattle given grass silage based diet. 2. Digestion of cell wall carbohydrates. Animal Feed Science and Technology, 33, 262-273.

Khazaal, K., M.T. Dentinho, J.M. Ribeiro & E.R. Ørskov (1993). A comparison of gas production during incubation with rumen contents *in vitro* and nylon bag degradability s predictors of the apparent digestibility *in vivo* and the voluntary intake of hays. Animal Production, 57, 105-112.

Korhonen M., A. Vanhatalo & P. Huhtanen (2002). Effects of protein source on amino acid supply, milk production, and metabolism of plasma nutrients in dairy cows fed grass silage. Journal of Dairy Science, 85, 3336-3351.

Kotb, A.R. & T.D. Luckey (1972). Markers in nutrition. Nutrition Abstracts & Reviews, 42, 813-845.

Kuoppala, K., M. Rinne, S. Ahvenjärvi, J. Nousiainen & P. Huhtanen (2004). Digestion kinetics of NDF in dairy cows fed silages from primary growth and regrowth of grass. In: The X International symposium on ruminant physiology, August 30th to September 4th 2004, Copenhagen, Denmark, short papers. Journal of Animal and Feed Sciences, 13, Supplement 1, 127-130.

Lechner-Doll, M., M. Kaske & W. von Engelhardt (1991). Factors affecting the mean retention time of particles in the forestomach of ruminants and camelids. In: T. Tsuda, Y. Sasaki & R. Kawashima (eds.) Physiological Aspects of Digestion and Metabolism in Ruminants, pp. 455-482.

Lewis, W.D., J.A Bertrand & T.C. Jenkins (1999). Interaction of tallow and hay particle size on ruminal parameters. Journal of Dairy Science, 82, 1532-1537.

Lindberg, J.E. (1981). The effect of basal diet on the ruminal degradation of dry matter, nitrogenous compounds and cell walls in nylon bags. Swedish Journal of Agricultural Research, 11, 159-169.

Lirette, A. & L.P. Milligan (1989). A quantitative model of reticulo-rumen particle degradation and passage. British Journal of Nutrition, 62, 465-479.

Llano, C.A. & E.J. DePeters (1985). Apparent digestibilities of diets varying in ratios of forage to concentrate and quality af forage at two intakes by dairy cows. Journal of Dairy Science 68, 1189-1197.

Lund, P. (2002). The effect of forage type on passage kinetics and digestibility of fibre in dairy cows. Ph.D. thesis, The Royal Veterinary and Agricultural University, Copenhagen. 169pp.

Mambrini, M. & J.L. Peyraud (1997). Retention time of feed particles and liquids in the stomach of ruminants and intestines of dairy cows. Direct measurements and calculations based on faecal collection. Reproduction Nutrition Development, 37, 427-442.

Matis, J.H. (1972). Gamma time-dependency in Blaxter´s compartment model. Biometrics, 28, 597-602.

McLeod, M.N. & D.J. Minson (1988). Large particle breakdown by cattle eating ryegrass and alfalfa. Journal of Animal Science, 66, 992-999.

Mertens, D.R. (1973). Application of theoretical mathematical models to cell wall digestion and forage intake in ruminants. Ph.D. thesis, Cornell Univeristy, Ithaca, NY. (Diss. Abstr. 74-10882).

Mertens, D.R. (1977). Dietary fibre components: relationship to the rate and extent of ruminal digestion. Federation Proceedings, 36, 187-192.

Mertens, D.R. (1993a). Kinetics of cell wall digestion and passage in ruminants. In: H.G. Jung, D.R. Buxton, R.D. Hatfield & J. Ralph (eds.) Forage Cell Wall Structure and Digestibility. American Society of Agronomy, pp. 535-570.

Mertens, D.R. (1993b). Rate and extent of digestion. In: J.M. Forbes & J. France (eds.) Quantitative Aspects of Ruminant Digestion and Metabolism. CAB International, Wallingford, pp. 13-50.

Mertens, D.R. (1994). Regulation of forage intake. In: G.C. Fahey, Jr., M. Collins, D.R. Mertens & L.E. Moser (eds.) Forage Quality, Evaluation and Utilization. American Society of Agronomy, pp. 450-493.

Mertens, D.R. & J.R. Loften (1980). The effect of starch on forage fibre digestion kinetics *in vitro*. Journal of Dairy Science, 63, 1437-1446.

Meyer, J.H.F. & R.I. Mackie (1986). Microbial evaluation of the intraruminal in sacculus digestion technique. Applied and Environmental Microbiology, 51, 622-629.

Michalet-Doreau, B., I. Fernandez & G. Fonty (2002). A comparison of enzymatic and molecular approaches to characterize the cellulolytic microbial ecosystems of the rumen and the caecum. Journal of Animal Science, 80, 790-796.

Moore, J.A., M.H. Poore & R.S. Swingle (1990). Influence of roughage source on kinetics of digestion and passage and on calculated extents if ruminal digestion in beef steers fed 65% concentrate diets. Journal of Animal Science, 68, 3412-3420.

Mould, F.L., E.R. Ørskov & S.O. Mann (1983). Associative effects of mixed feed. I Effects of type and level of carbohydrate supplementation and the influence of rumen fluid pH on cellulolysis *in vivo* and dry matter digestion of various roughage. Animal Feed Science and Technology, 10, 15-30.

Murphy, M.R. & P.M. Kennedy (1993). Particle dynamics. In: J.M. Forbes & J. France (eds.) Quantitative Aspects of Ruminant Digestion and Metabolism. CAB International, pp. 87-105.

Nocek, J. E. (1988). *In situ* and other methods to estimate ruminal protein and energy digestibility: a review. Journal of Dairy Science, 71, 2051–2069.

Nørgaard, P. & A. Sehic (2003). Particle size distribution in silage, boluses, rumen content and faeces from cows fed grass silage with different theoretical chopping length. The Sixth International Symposium on the Nutrition of Herbivores. Tropical and Subtropical Agroecosystems, 3, 457-460.

Nousiainen, J., S. Ahvenjärvi, M. Rinne, M. Hellämäki & P. Huhtanen (2004). Prediction of indigestible cell wall fraction of grass silage by near infrared reflectance spectroscopy. Animal Feed Science and Technology, 115, 295-311.

Nousiainen, J., M. Rinne, M. Hellämäki & P. Huhtanen (2003a). Prediction of the digestibility and D-value of primary growth grass silages harvested at different stages of maturity from various laboratory measurements. Animal Feed Science and Technology, 103, 97-111.

Nousiainen, J., M. Rinne, M. Hellämäki & P. Huhtanen (2003b). Prediction of intake of primary growth and regrowth silages from chemical composition, pepsin-cellulase solubility and indigestible cell wall content. Animal Feed Science and Technology, 110, 61-74.

Nozière, P. & B. Michalet-Doreau (1996). Validation of *in sacco* method: influence of sampling site, nylon bag or rumen contents, on fibrolytic activity of solid-associated microorganisms. Animal Feed Science and Technology, 57, 203-210.

Nozière, P. & B. Michalet-Doreau (2000). *In sacco* methods. In: J.P.F. D´Mello (ed.) Farm Animal Metabolism and Nutrition. CAB International, Oxon, pp. 233-253.

NRC (National Research Council) (2001). Nutrient requirements of dairy cattle, 7th revised edition, National Academy Press, Washington, DC. 381p.

Oba, M. & M.S. Allen (2003). Effects of corn grain conservation method on ruminal digestion kinetics for lactating dairy cows at two dietary starch concentrations. Journal of Dairy Science, 86, 184-194.

Offer, N.W. & J. Dixon (2000). Factors affecting outflow rate from the reticulo-rumen. Nutrition Abstracts and reviews. Series B. Livestock Feeds and Feeding, 70, 833-844.

Okine, E.K. & G.W. Mathison (1991). Effects of feed intake on particle distribution, passage of digesta, and extent of digestion in the gastrointestinal tract of cattle. Journal of Animal Science 69, 3435-3445.

Oldham, J.D. (1984). Protein-energy interrelationships in dairy cows. Journal of Dairy Science, 67, 1090-1114.

Ørskov, E.R. (1982). Protein Nutrition in Ruminants. Academic Press. New York. 160p.

Ørskov, E.R. & I. McDonald (1979). The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. Journal of Agricultural Science, Cambridge, 92, 499-503.

Owens, F.N. & A.L. Goetch (1986). Digesta passage and microbial protein synthesis. In: L.P. Milligan, W.L. Grovum & A. Dobson (eds.) Control of Digestion and Metabolism in Ruminants. Prentice-Hall, Englewood Cliffs, NJ, USA, pp. 196-223.

Paloheimo, L. & A. Mäkelä (1959). Further studies on the retention time of food in the digestive tract of cows. Acta Agralia Fenniae, 94, 15-39.

Pell, A.N & P. Schofield (1993). Computerized monitoring of gas production to measure forage digestion *in vitro*. Journal of Dairy Science, 76, 1063-1073.

Pond, K.R., W.C. Ellis, J.H. Matis, H.M. Ferreiro & J.D. Sutton (1988). Compartmental models for estimating attributes of digesta flow in cattle. British Journal of Nutrition, 60, 571-595.

Poppi, D.P., W.C. Ellis, J.H. Matis & C.E. Lascano (2001). Marker concentration patterns of labelled leaf and stem particles in the rumen of cattle grazing bermuda grass (*Cynodon dactylon)* analysed by reference to a draft model. British Journal of Nutrition, 85, 553-563.

Poppi D.P., D.J. Minson & J.H. Ternouth (1981). Studies of cattle and sheep eating leaf and stem fractions of grasses. III The retention time in the rumen of large feed particles. Australian Journal of Agricultural Research, 32, 123-137.

Poppi, D.B., B.W. Norton, D.J. Minson & R.W. Hendricksen (1980). The validity of the critical particle size theory for particles leaving the rumen. Journal of Agricultural Science, 94, 275-280.

Rinne, M. (2000). Influence of the timing of the harvest of primary grass growth on herbage quality and subsequent digestion and performance in the ruminant animal. Academic Dissertation. University of Helsinki, Department of Animal Science, publications 54, 42 p.+5 encl.

Rinne, M., P. Huhtanen & S. Jaakkola (1997). Grass maturity effects on cattle fed silage-based diets. 2. Cell wall digestibility, digestion and passage kinetics. Animal Feed Science and Technology, 67, 19-35.

Rinne, M., P. Huhtanen & S. Jaakkola (2002). Digestive processes of dairy cows fed silages harvested at four stages of maturity. Journal of Animal Science, 80, 1986-1998.

Rinne, M. & A. Nykänen (2000). Timing of primary growth harvest affects the yield and nutritive value of timothy-red clover mixtures. Agricultural and Food Science in Finland, 9, 121-134.

Robinson, P.J., J.G. Fadel & S. Tamminga (1986). Evaluation of mathematical models to describe neutral detergent residue in terms of its susceptibility to degradation in the rumen. Animal Feed Science and Technology, 15, 249-271.

Robinson, P.J., S. Tamminga & A.M. van Vuuren (1987). Influence of declining level of feed intake and varying proportion of starch in the concentrate on rumen ingesta quantity, composition and kinetics of ingesta turnover in dairy cows. Livestock Production Science, 17, 37-62.

Robinson, P.H., M. Gill & J.J. Kennelly (2002). Influence of time of feeding a protein meal on diurnal patterns of amino acids in duodenal digesta protein of lactating dairy cows. Animal Feed Science and Technology, 97, 115-121.

Rooke, J.A., N.H. Lee & D.G. Armstrong (1987). The effects of intraruminal infusions of urea, casein, glucose syrup and a mixture of casein and glucose syrup on nitrogen digestion in cattle receiving sucrose grass silage diets. British Journal of Nutrition, 57, 89-98.

Russell, J.B. & D.B. Dombrowski (1980). Effect of pH on the efficiency of growth by rumen bacteria in continuous culture. Applied and Environmental Microbiology, 39, 604-610.

Satter, L.D., H.G. Jung, A.M van Vuuren & F.M. Engels (1999). Challenges in the nutrition of high-producing ruminants. In: H.J.G. Jung & G.C. Fahey, Jr. (eds.) Nutritional Ecology of Herbivores. Proceedings of the Vth international symposium on the nutrition of herbivores. pp. 609-646.

Sauvant, D., J. Dijkstra & D. Mertens (1995). Optimisation of uminal digestion: a modelling approach. In: M. Journet, E. Grenet, M.-H. Farge, M. Thériez & C. Demarquilly (eds.) Recent Developments in the Nutrition of Herbivores. INRA Editions, Paris, pp. 143-165.

Sauvant, D., R. Baumont & P. Faverdin (1996). Development of a mechanistic model of intake and chewing activities of sheep. Journal of Animal Science, 74, 2785-2802.

Schofield, P. (2000). Gas production methods. In: J.P.F. D´Mello (ed.) Farm Animal Metabolism and Nutrition. CAB International, Oxon, pp. 209-232.

Schofield, P. & A.N. Pell (1995). Measurement of kinetic analysis of the neural detergent soluble fraction carbohydrate fraction of legumes and grasses. Journal of Animal Science, 73, 3455-3463.

Shaver, R.D., A.J. Nytes, L.D. Satter & N.A. Jorgensen (1986). Influence of amount of feed intake and forage physical form on digestion and passage of prebloom alfalfa hay. Journal of Dairy Science, 69, 1545-1559.

Shingfield, K.J., A. Vanhatalo & P. Huhtanen (2003). Comparison of heat-treated rapeseed expeller and solvent-extracted soybean meal protein supplements for dairy cows given grass silage-based diets. Animal Science, 77, 305-317.

Siddons, R.C., J. Paradine, D.E. Beever & P.R. Cornell (1985). Ytterbium acetate as particulate-phase marker in the rumen. British Journal of Nutrition, 54, 509-519.

Smith, L.W. (1989). A review of intrinsically ^{14}C and rare-earth-labelled neutral detergent fiber to estimate particle digestion and passage. Journal of Animal Science 67, 2123-2128.

Smith, L.W., H.K. Goering & C.H. Gordon (1972). Relationships of forage compositions with rates of cell wall digestion and indigestibility of cell walls. Journal of Dairy Science, 55, 1140-1147.

Sniffen, C.J., J.D. O´Conner, P.J. van Soest, D.G. Fox & J.B. Russel (1992). A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. Journal of Animal Science, 70, 3562-3577.

Staples, C.R., R.L. Fernando, G.C. Fahey, L.L. Berger Jr. & E.H. Jaster (1984). Effects of intake of mixed diet by dairy steers on digestion events. Journal of Dairy Science 67, 995-1006.

Stensig T. & P.H. Robinson (1997). Digestion and passage kinetics of forage fiber in dairy cows as affected by fiber-free concentrate in the diet. Journal of Dairy Science, 80, 1339-1352.

Stensig, T., M.R. Weisbjerg & T. Hvelplund (1998). Digestion and passage kinetics in dairy cows as affected by the proportion of wheat starch or sucrose in the diet. Acta Agriculturae Scandinavica. Section A Animal Science, 48, 129-140.

Stern, M. D., A. Bach & S. Calsamiglia. (1997). Alternative techniques for measuring nutrient digestion in ruminants. Journal of Animal Science, 75, 2256–2276.

Sutherland, T.M. (1988). Particle separation in the forestomach of sheep. In: A. Dobson & M.J. Dobson (eds.) Aspects of Digestive Physiology in Ruminants. Cornell University Press, Ithaca, NY, pp. 43-73.

Tamminga, S. (1993). Influence of feeding management on ruminant fibre digestibility. In: H.G. Jung, D.R. Buxton, R.D. Hatfield & J. Ralph (eds.) Forage Cell Wall Structure and Digestibility. American Society of Agronomy, pp. 571-602.

Tamminga, S., P.H. Robinson, M. Vogt & H. Boer (1989). Rumen ingesta kinetics of cell wall components in dairy cows. Animal Feed Science and Technology 25, 1-2, 89-98.

Tamminga, S. & A.M. van Vuuren (1988). Formation and utilisation of end products of lignocellulose degradation in ruminants. Animal Feed Science and Technology 21, 141-159.

Tesfa, A.T. (1992). Effects of rapeseed oil on rumen enzyme activity and *in sacco* degradation of grass silage. Animal Feed Science and Technology, 36, 77-89.

Tesfa, A.T. (1993). Effects of rape-seed oil supplementation on digestion, microbial protein synthesis and duodenal microbial amino acid composition in ruminants, Animal Feed Science and Technology, 41, 313-328.

Theodorou, M.K., B.A. Williams, M.S. Dhanoa, A.B. McAllan & J. France (1994). A simple gas production method using a pressure transducer to determine fermentation kinetics of ruminant feeds. Animal Feed Science and Technology 48, 185-197.

Tothi, R., P. Lund, M.R. Weisbjerg & T. Hvelplund (2003). Effect of expander processing on fractional rate of maize and barley starch degradation in the rumen of dairy cows estimated using rumen evacuation and *in situ* techniques. Animal Feed Science and Technology, 104, 71-94.

Udén, P. (1984). Digestibility and digesta retention in dairy cows receiving hay or silage at varying concentrate levels. Animal Feed Science and Technology, 11, 279-291.

Udén, P., P.E. Colucci & P.J. van Soest (1980). Investigation of chromium, cerium and cobalt as digesta flow markers in the rate of passage studies. Journal of Science and Food Agriculture 31, 625-632.

Ueda, K., A. Ferlay, J. Chabrot, J.J. Loor, Y. Chillard & M. Doreau (2003). Effect of linseed oil supplementation on ruminal digestion in dairy cows fed diets with different forage:concentrate ratios. Journal of Dairy Science, 86, 3999-4007.

Ulyatt, M.J., D.W. Dellow, A. John, C.S.W. Reid, G.C. Waghorn, L.P. Milligan, W.L. Grovum & A. Dobson (1986). Contribution of chewing during eating and rumination to the clearance of digesta from the ruminoreticulum. In: L.P. Milligan, W.L. Grovum & A. Dobson (eds.) Control of Digestion and Metabolism in Ruminants. Prentice-Hall, Englewood Cliffs, NJ, USA, pp. 498-515.

Van Milgen, J., L.L. Berger & M.R. Murphy (1993). Digestion kinetics of alfalfa straw assuming heterogeneity of the potentially digestible fraction. Journal of Animal Science, 71, 1917-1923.

Van Milgen, J., M.R. Murphy & L.L. Berger (1991). A compartmental model to analyse ruminal digestion. Journal of Dairy Science, 74, 2515-2529.

Van Soest, P.J. (1963). Use of detergents in the analysis of fibrous feeds. I. Preparation of fiber residues of low nitrogen content. Journal of the Association of Official Analytical Chemists 46, 825-829.

Van Soest, P.J. (1994). Nutritional Ecology of Ruminants, second edition, Cornell University Press, Ithaca, USA, 476pp.

Van Soest, P.J., M.E. Van Amburg & L.O. Tedeschi (2000). Rumen balance and rates of fiber digestion. Cornell Nutrition Conference for Feed Manufacturers. Proceedings 2000. pp. 150-166.

Vega, A. De & D.P. Poppi (1997). Extent of digestion and rumen conditions as factors affecting passage of liquid and digesta particles in sheep. Journal of Agricultural Science, Cambridge, 128, 207-215.

Voelker, J.A. & M.S. Allen (2003). Pelleted beef pulp substituted for high-moisture corn: 2. Effects on digestion and rumen digestion kinetics in lactating dairy cows. Journal of Dairy Science, 86, 3553-3561.

Volden, H. (1999). Effects of level of feeding and ruminally undegraded protein on ruminal bacterial protein synthesis, escape of dietary protein, intestinal amino acid profile, and performance of diary cows. Journal of Animal Science, 77, 1905-1918.

Waldo, D.R. (1970). Factors influencing the voluntary intake of forages. In: R.F. Barnes *et al*. (eds.) Proceedings of the National Conference on Forage Quality, Evaluation and Utilisation, pp. E1-22.

Waldo, D.R., L.W. Smith & E.I. Cox (1972). Model of cellulose disappearance from the rumen. Journal of Dairy Science, 55, 125-129.

Weisbjerg, M.R. & C.F. Børsting (1989). Influence of fat and feed level on fibre digestibility *in vitro* and in sacco and on volatile fatty acid proportions in the rumen. Acta Veterinaria Scandinavica, Supplement 86, 137-139.

Weisbjerg, M.R., H. Gado, T. Hvelplund & B.B. Jensen (1999). The effect of easily fermentable carbohydrates and pH on fibre digestibility and VFA pattern in an *in vitro* continuous culture system. South African Journal of Animal Science, ISRP, 29, 112-113.

Weisbjerg, M.R., T. Hvelplund, V.F. Kristensen & T. Stensig (1998). The requirement for rumen degradable protein and the potential for nitrogen recycling to the rumen in dairy cows. In: Proceedings of the 25th Scientific Conference AICC – Arusha , August 5-7, 1998. TSAP Conferences Series, 25, 110-118.

Weisbjerg, M.R., T. Hvelplund & K. Søegaard (2004a). Prediction of digestibility neutral detergent solubles using the Lucas principle. Journal of Animal and Feed Sciences 13, Supplement 1, 239-242.

Weisbjerg, M. R. T. Hvelplund T. & K. Søegaard (2004b). Prediction of NDF digestibility based on assumptions about true digestibility and endogenous loss of NDS. Journal of Animal and Feed Sciences 13, Supplement 1, 243-246.

Weisbjerg, M.R., M. Mikkelsen, D. Bossen & P. Lund (2003). Estimation of potential degradability and rate of degradation of NDF from barley and wheat whole crop. In: T.H. Garmo (ed.) Symposium Proceedings, Early harvested forage in milk and meat production, 23-24 October 2003. Kringler, Nannestad, Norway, pp. 114-116.

Weiss, W.P. (1994). Estimation of digestibility of forages by laboratory methods. In: G.C. Fahey, Jr., M. Collins, D.R. Mertens & L.E. Moser (eds.) Forage Quality, Evaluation and Utilization. American Society of Agronomy, pp. 644-681.

Welch, J.G. (1982). Rumination, particle sizes and passage from the rumen. Journal of Animal Science, 54, 885-894.

Wilkins, R.J. (1969). The potential digestibility of cellulose in forages and faeces. Journal of Agricultural Science, Cambridge, 83, 57-64.

Williams, A.G. & S.E. Withers (1982). The production of plant cell wall polysaccharide-degrading enzymes by rumen isolates grown on a range of carbohydrate substrates. Journal of Applied Bacteriology 52, 377-387.

Wilman, D., G.R. Foulkes & D.L. Givens (1996). The rate and extent of cell-wall degradation *in vitro* for 40 silages varying in composition and digestibility. Animal Feed Science and Technology, 63, 111-122.

Wylie, M.J., W.C. Ellis, J.H. Matis, E.M. Bailey, W.D James & D.E. Beever (2000). The flow of forage particles and solutes through segments of the digestive tract of cattle. British Journal of Nutrition 83, 295-306.

Part II: Absorption mechanisms

Transport systems in the epithelia of the small and large intestines

G. Breves[1] and S. Wolffram[2]
[1]Institute of Physiology, School of Veterinary Medicine, Bischofsholer Damm 15/102, 30173 Hannover, Germany
[2]Institute of Animal Nutrition and Physiology, University of Kiel, 24098 Kiel, Germany

Abstract

The present state of epithelial mechanisms of intestinal sugar, amino acid, peptide and phosphate transport in ruminants is summarized. For sugar absorption SGLT1 and GLUT5 have been identified for luminal uptake of monosaccharides and GLUT2 is expressed at the basolateral membrane for sugar extrusion. Sugar transport has gained special interest in lactating cows in order to improve energy intake. For amino acid absorption similar mechanisms as for monogastric species have to be assumed. Recently, PEPT1 has been identified to be involved in absorption of di- and tripeptides in bovine intestinal tract. Small ruminants have been investigated in detail for intestinal P_i absorption, and it was concluded that both, a H^+-dependent system and a Na^+-coupled system, are expressed in the duodenum and jejunum, respectively. Only the jejunal system responds to changes in dietary P intake.

Keywords: intestinal absorption, sugars, amino acids, peptides, phosphate

Introduction

In recent years substantial progress has been made regarding the different epithelial mechanisms which are involved in intestinal transport processes and this is mainly true for the small intestines. As in monogastric animals end products of enzymatic digestion of proteins, fats, nucleic acids and nucleotides are mainly absorbed from the small intestines. Many of these processes are mediated by coupled mechanisms and may thus include electrolyte absorption. From a number of recent studies in lactating cows, there is increasing evidence that most of the concepts regarding carbohydrate metabolism have to be changed substantially. Under certain dietary conditions substantial amounts of starch will flow into the small intestines and will thus contribute to glucose metabolism by intestinal glucose absorption. However, this is an area with a high demand for further research especially regarding potentially limiting factors. In addition to the forestomach epithelium, short chain fatty acids originating from hindgut microbial fermentation can be absorbed from the hindgut epithelium. This has been reviewed in detail by Rechkemmer *et al.* (1995) and will not further be considered as the state of knowledge has not substantially changed since then. It is the major aim of this review to summarize the relevant epithelial mechanisms of intestinal absorption of monosaccharides, amino acids and peptides as major nutrients and of phosphate as an electrolyte with substantial progress in recent years how intestinal absorption is mediated.

Carbohydrate digestion and monosaccharide absorption

In contrast to monogastric animals, it was generally assumed for fully developed ruminants that due to microbial fermentation in the forestomach region hardly any monosaccharides would reach the small intestines for subsequent absorption (Bassett, 1975). Weaning was shown to be the major factor to decrease the capacity of the small intestines for glucose absorption (Scharrer, 1976; Scharrer *et al*., 1979). However, this concept was changed again when it could be demonstrated in ruminating sheep that the infusion of either D-glucose or methyl-α-D-glucopyranoside into the duodenum resulted in activation of Na^+-dependent D-glucose transport to a level similarly as it had been found in pre-ruminant sheep (Shirazi-Beechey *et al*., 1991b; Lescale-Matys *et al*., 1993). This led to the conclusion that even in ruminating sheep intestinal glucose transport may occur when the substrate reaches the small intestines. Whereas most of these findings were obtained from studies in small ruminants, intestinal starch digestion has become a topic of major interest in high yielding cows with regard to energy metabolism and improvement of feed efficiency. In these animals grain based concentrates provide high amounts of starch which potentially may escape rumen fermentation and may thus reach the small intestines for enzymatic digestion. The proportion of ruminal starch digestion is still under discussion. There is a huge number of factors influencing rumen starch digestion such as starch source, degree of plant maturation, feed technology, feed intake and feed particle size. These factors have been reviewed in detail by Mills *et al*. (1999a). According to literature data they found a range for starch intake in lactating cows between 3.5 and 8.6 kg/day from different grain sources with respective values for rumen digestibility between 47 and 89 %. From rumen processes it can be concluded that small intestinal starch digestion and monosaccharide absorption will be determined by the following criteria:

1. Rate of flow of starch into the small intestines.
2. Enzymatic hydrolysis.
3. Epithelial transport of sugars.

Rate of flow of starch into the small intestines

The high variability of flow of starch into the small intestines as affected by the source of starch is exemplarily shown in Table 1 which summarizes data from Sutton and Oldham as published by Reynolds *et al*. (1997).

Table 1. Passage of starch throughout the gastrointestinal tract of 4 lactating dairy cows (Source: Sutton and Oldham as published by Reynolds et al.*, 1997).*

	Barley 60%	Barley 90%	Maize 60%	Maize 90%
Starch passage (kg/day)				
Intake	4.0	5.5	4.4	6.4
Duodenum	0.6	0.8	2.2	3.5
Ileum	0.1	0.2	0.6	1.1
Faeces	0.1	0.1	0.2	0.7

In these experiments overall starch digestibility was slightly higher for barley than for maize, however, flow of starch into and net disappearance from the small intestines was significantly higher for maize. When the net disappearance from the small intestines was calculated as percentage of starch entering the duodenum which indicates the efficiency of net absorption, the average value for both barley diets was 79 % in comparison with 70 % for both maize diets. This might be interpreted as an indication for a limited capacity for starch digestion in the small intestines. Flow rates of starch into the duodenum between 0.5 and 3.5 kg/day are in accordance with data obtained from many other studies. According to McCarthy *et al.* (1989), the maximal amount of maize starch entering the duodenum and subjected to hydrolysis and absorption can be around 4.6 kg/day.

Enzymatic hydrolysis

As in monogastric species intestinal starch digestion is mediated by different enzymes such as pancreatic α-amylase and brush border membrane bound maltase and isomaltase (Mills *et al.*, 1999b). In contrast to monogastric species, no sucrase activity could be determined (Harmon, 1992). The pH optimum for α-amylase is around 6.9 and for maltase and isomaltase between 5.8 and 6.2, respectively. This might be disadvantageous when the low pH values in the upper small intestines of ruminants are considered, especially since for α-amylase differences of about 0.5 from pH optimum resulted in changes of activity of up to 20 % (Owens *et al.*, 1986). The quantitative distribution of disaccharidase activity along the intestinal axis is not yet fully understood. In calves the lowest activities for maltase and isomaltase were found in the duodenum whereas the highest activities were present in the mid jejunum and ileum (Kreikemeier *et al.*, 1990). In contrast, a decline in activity from the duodenum to the ileum has been described by Nocek and Tamminga (1991) in cows. Increases in energy intake resulted in higher specific activities of pancreatic amylase with no changes in maltase activity (Russell *et al.*, 1981; Kreikemeier *et al.*, 1990). However, starch infusions into the abomasum resulted in decreases of pancreatic α-amylase concentration (Walker and Harmon, 1995). They discussed regulatory peptides such as peptide YY as mediators for the negative feedback, however, the biochemical concept regulating enzyme secretion has not yet been clarified. Other inhibitory dietary factors for α-amylase are lignin and tannin derivatives which can be associated with starch granules (Owens *et al.*, 1986). The potential limitations of intestinal starch digestion at the enzyme level are still under debate. Huntington (1997) has discussed the α-amylase as the most relevant limiting enzyme for starch hydrolysis rather than the brush border membrane enzymes.

Epithelial transport of sugars

Intestinal sugar absorption can principally be mediated by passive and secondary active processes. For passive transport either the paracellular or the transcellular pathway may be used. Evidence for paracellular glucose transport has been obtained from studies in rats and has introduced a rather controversial discussion on its biological significance (Pappenheimer and Reiss, 1987). So far this route has not been investigated for ruminants in detail, however, there is still no experimental evidence that this pathway is of major relevance in ruminants. Therefore, it will not be considered further in this review. For facilitated diffusion of fructose across the apical membrane of jejunal enterocytes, the GLUT5 has been identified for different

species such as rats and rabbits (Thorens, 1996). The GLUT5 mRNA has also been detected in all gastrointestinal segments except the abomasum of lactating dairy cows. The functional properties for ruminants, however, have not yet been clarified (Zhao *et al*., 1998). For secondary active glucose and galactose transport, the SGLT1 has been identified for many vertebrate species including ruminants (Hediger and Rhoads, 1994; Shirazi-Beechey *et al*., 1995; Zhao *et al*., 1998). It is energized by the basolateral Na^+/K^+ATPase and from stoichiometry of both components it can be calculated that three glucose molecules are transported per two ATP hydrolysed in the enterocyte. The SGLT1 is a high affinity transport system with a great deal of homology (80 % or more) between species. The transporter density decreases along the intestinal axis and in the upper intestinal parts the number of transporters per enterocyte is estimated to be around 10^5 – 10^7. The sodium:glucose ratio per cycle is 2:1 and its K_m ranges between 0.1 and 0.5 mmol/l. Both parameters differentiate the SGLT1 from its renal isoform SGLT2 which, in addition, can only transport glucose (Hediger and Rhoads, 1994). In ruminants, Shirazi-Beechey and co-workers have been the first to demonstrate age- and developmental-dependent differences in SGLT1 expression in the ovine intestinal tract. They could demonstrate that the activity of the SGLT1 protein in the intestinal brush border membrane was high in the pre-ruminant lamb, decreased substantially in association with development of rumen functionality and could be stimulated again when D-glucose or derivatives were introduced into the intestinal lumen (Shirazi-Beechey *et al*., 1995). The expression of SGLT1 mRNA and SGLT1 protein differed with regard to distribution along the crypt-villus axis. Whereas most of the mRNA was found in the crypt region the protein was detected in the villus region. With these findings they have developed an appropriate model for studying the principles of nutrient regulation of transport systems. On the basis of their studies the SGLT1 mRNA expression has also been detected for all segments of the bovine gastrointestinal tract and SGLT1 protein expression could be demonstrated for jejunal and ileal brush border membranes of lactating cows (Zhao *et al*., 1998). With respect to feeding behaviour, ruminants can be divided into grass and roughage type (e. g. cow and sheep), intermediate type (e. g. goat and fallow deer) and concentrate selecting type animals (e. g. moose and roe deer). Comparative experiments on SGLT1 expression have shown that in contrast to sheep and cows, the level of SGLT1 mRNA and SGLT1 protein expression remained significantly high in intermediate and concentrate selecting type animals which can be correlated with the availability of monosaccharides in the intestinal tract in these animals due to feeding behaviour (Wood *et al*., 2000).

For basolateral extrusion of glucose, galactose and fructose by facilitated diffusion, the GLUT2 has been identified as a low affinity system (Thorens, 1996). So far it has not been demonstrated for ruminants that glucose can be transported by GLUT2 in the apical and basolateral membrane at very high luminal glucose concentrations as it has been found for rat jejunum (Helliwell and Kellett, 2002).

Substrate induction of sugar transport has been detected as an important principle of regulation. Expression of SGLT1 was induced when D-isomers of glucose, galactose, fructose, mannose and xylose were present at the luminal side of the tissues. This was in contrast to induction of GLUT2 by high glucose, galactose or fructose diets and induction of GLUT5 by high fructose diets (Hediger and Rhoads, 1994; Thorens, 1996). With regard to turnover time of enterocytes along the crypt-villus axis, the functional SGLT1 protein could only be detected in mature enterocytes

from the upper villus region after 3 days of D-glucose infusion and reached its maximum after 4 days (Shirazi-Beechey *et al*., 1995). From luminal induction of SGLT1 the concept of the glucose sensor has been developed. There is experimental evidence that the sensor is externally localized in the crypt region and consists of a G protein coupled receptor and the cAMP/protein kinase A pathway (Dyer *et al*., 2003). In addition to substrate induction, other nutrients such as casein have been found as potential modulators. By abomasal infusion of casein, Mabjeesh *et al*. (2003) could demonstrate an increase in digestibility of non-structural carbohydrates and in V_{max} of SGLT1 in the mid jejunum of lambs. Since proteins can induce pancreatic enzyme secretion, it cannot be differentiated whether the SGLT1 stimulation was a direct or indirect effect.

Evidence for regulation by different peptides such as epidermal growth factor, transforming growth factor α, peptide YY, insulin like growth factors, somatotropin and somatostatin and others has been obtained from different studies, their relevance for ruminants, however, has still to be clarified (Bird *et al*., 1996).

Gastrointestinal absorption of amino acids and peptides

As in monogastric species, nitrogen is taken up with feed in the form of protein and non-protein nitrogenous (NPN) compounds, including - amongst others - free amino acids, ammonia and urea. What is different in ruminant animals as compared to monogastric species is the fact that ruminants are only partly dependent on dietary protein quality, e.g., a balanced intake of dispensable and indispensable amino acids. This is due to the fact that the major part of dietary protein and NPN substances are finally transformed within the forestomachs to microbial protein which in turn is the major nitrogenous fraction arriving at the duodenum.

Despite these peculiarities, present knowledge indicates no substantial differences with respect to either the absorptive site or the principal mechanisms involved in the absorption of absorbable products deriving from enzymatic protein digestion. Thus, as in monogastric species, α-amino nitrogen, either in the form of free amino acids (AAs) or small peptides (mainly tri- and dipeptides) is principally absorbed in the small intestine. Although some absorption of free amino acids may occur from the forestomachs and hindgut (Krehbiel and Matthews, 2003), these sites can be considered of minor importance for overall AA absorption. With respect to peptide absorption, several studies indicate a major contribution of the forestomachs to peptide-bound AAs. This interpretation, however, is almost exclusively based on calculated net fluxes across the portal-drained (forestomachs, cranial abomasum, spleen and pancreas) and mesenteric-drained (small intestine, caecum, colon, mesenteric fat and pancreas) viscera (Krehbiel and Matthews, 2003) with no convincing evidence for the existence of functionally active carrier-mediated mechanisms for AA and peptides in the forestomachs (Martens *et al.*, 2001) and large intestine (Binder, 1970).

In the following, present knowledge regarding the absorption of AAs and peptides will be shortly reviewed with emphasis on the transport systems involved. Most of the pertinent information, however, stems from experiments with cell cultures or tissues derived from monogastric species with only relatively few papers considering this important aspect specifically in ruminant animals. Net-fluxes of free and peptide-bound AAs across the portal drained viscera will not be covered by this contribution. Several recent comprehensive review articles dealing with the gastrointestinal

handling of AAs and peptides in various species including farm animals are available for the more deeply interested reader (Barker and Ellory, 1990; Mailliard *et al*., 1995; Ganapathy *et al*., 2001; Matthews, 2000; Krehbiel and Matthews, 2003).

Absorption of amino acids

Irrespective of the species considered, AAs can principally be absorbed by the paracellular and transcellular route, whereby transcellular uptake can be further differentiated into passive (no specific carrier involved) and carrier-mediated processes including active and facilitated transport mechanisms (Barker and Ellory, 1990; Matthews, 2000). In contrast to passive and facilitated diffusional processes, an active transport mechanism is capable of transporting substrates against a concentration gradient. Basically, carrier-mediated transcellular mechanisms are of increasing importance for an efficient amino acid absorption at decreasing substrate levels within the intestinal contents (Barker and Ellory 1990; Matthews, 2000). Interestingly, the basolateral membrane of enterocytes appears to have a higher passive permeability for free amino acids as compared to the luminal brush-border membrane. Thus, for instance, studies using isolated membrane vesicles derived from the luminal and antiluminal poles of enterocytes indicate a 4.5 times higher passive permeability for L-alanine at the basolateral membrane compared to the brush-border membrane (Stevens *et al*., 1984). Findings with regard to passive permeability of membranes, however, obtained with isolated membrane vesicles, should be interpreted carefully because alterations of membrane characteristics during the isolation of those membranes may occur during preparation.

As in monogastric species, transcellular AA absorption from the gastrointestinal tract in ruminants mainly occurs from the small intestine which is therefore equipped with a multitude of AA carriers differing with respect to substrate specificity and membrane distribution. In this context, it is important to keep in mind that the epithelial cells, lining the mucosal surface, are so-called polarized cells with distinct patterns of carrier proteins integrated in the brush-border membrane (luminal) and the so-called basolateral membrane (antiluminal), respectively. This principle, which can be found in several absorbing and secreting epithelia, is a prerequisite for vectorial transport of substrates from the intestinal contents into the systemic circulation. Until now, however, AA carriers in intestinal tissues from ruminant species have not been well characterized with respect to substrate specificity and membrane distribution (Matthews, 2000). In contrast to pigs and other monogastrics, in sheep and cattle the distal parts of the small intestine appear to possess a greater potential for the absorption of free AAs (Matthews, 2000).

Carrier-mediated uptake of free AAs in the small intestine in monogastric and ruminant species is mediated by an orchestra of several Na^+-dependent and Na^+-independent transport proteins with significant overlap in their substrate specificity. Transport systems identified so far for neutral, cationic, anionic and imino acids in the small intestine are summarized in Table 2.

Although the expression of all of these transporters has been confirmed on the mRNA level, much work has to be done to identify the respective proteins. Furthermore, expression levels as well as expression patterns in the brush-border and basolateral membranes can be expected to be modified according to AAs (protein) nutrition and physiological state of an animal (see below).

Table 2. Amino acid transport systems of the mammalian small intestine (Sources: Ganapathy et al.*, 2001, Krehbiel and Matthews, 2003 and Mailliard* et al.*, 1995).*

Transport system	Specific transporter	Transport substrates	Membrane location	Coupled ions
A	ATA2	Neutral AAs	BLM	Na^+
B^0	ASCT2		BBM	Na^+
Asc	Asc-1		BLM	–
L	LAT2		BLM	–
N	SN2		BBM or BLM	Na^+
T	TAT1		BLM	–
IMINO	unknown		BBM	Na^+, Cl^-
$B^{0,+}$	$ATB^{0,+}$	Neutral and	BBM	Na^+, (Cl^-)
y^+L	y^+LAT1	cationic AAs	BLM	Na^+ (only neutral AAs)
	y^+LAT2		BLM	
$b^{0,+}$	BAT1		BBM	–
	$b^{0,+}AT$		BBM	–
	4F2-lc6		BBM	–
y^+	CAT1	Cationic AAs	BBM/BLM	–
X^-_{AG}	EAAT2		Not known	Na^+, K^+
	EAAT3	Anionic AAs	Not known	Na^+, K^+
x_c^-	xCT		Not known	-

BLM = basolateral membrane
BBM = brush-border membrane

The complexity and functional interconnections between various AA carriers can be deduced from the model proposed by Chillaron *et al.* (1996). In this model (Figure 1), dipolar (neutral) amino acids are taken up against their gradient via a Na^+/AA^0 symporter named B^0. This secondary active mechanism is energized by the inwardly directed transmembrane electrochemical Na^+ gradient which in turn is maintained by the Na^+/K^+-ATPase exclusively located in the basolateral enterocyte membrane. The gradient of neutral AAs directed from the cytosol to the extracellular compartment is then used to drive uptake of cationic AAs and cystine against their concentration gradients via the $b^{0,+}$ antiporter located in the brush-border membrane. Cationic and neutral AAs (including cysteine produced by glutathion-dependent intracellular reduction of cystine) are proposed to leave the enterocyte via systems y^+L and L, respectively, in the basolateral membrane. Taken together, transepithelial absorption of cationic amino acids would thus be driven by the Na^+ gradient (Van Winkle, 2001).

With respect to dietary regulation of AA transport in the small intestine, only the influence of dietary protein or dietary AAs will be shortly addressed here. Dietary AA induces AA transport activity in the brush-border membrane of enterocytes. Moreover, the characteristics of much of this transport resemble those of the Na^+-independent system $b^{0,+}$, which is associated with BAT1 expression. Although some intestinal transport is Na^+-dependent, and hence dissimilar from system $b^{0,+}$ (a Na^+-independent system), data reported recently are consistent with the

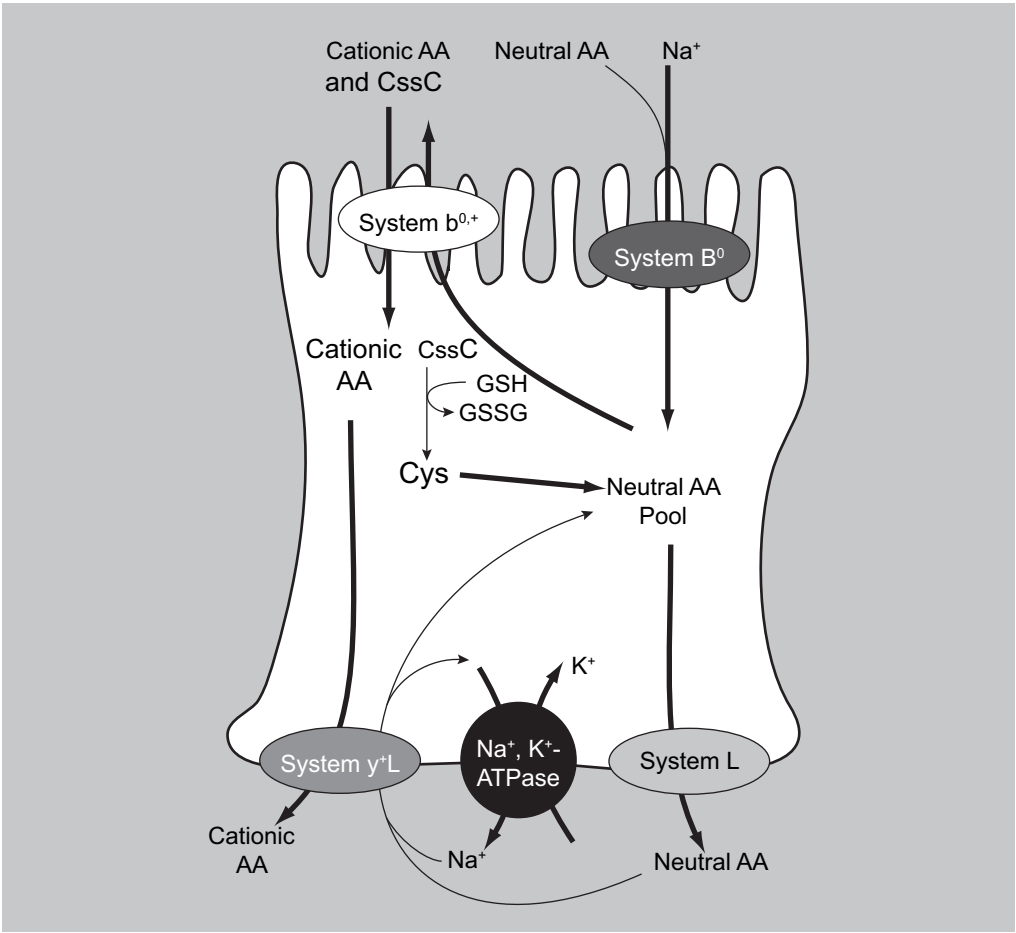

Figure 1. Model for the absorption of cationic AAs and cystine and its coupling to Na^+-dependent absorption of neutral AAs (Source: adapted from Van Winkle, 2001).
CssC = cystine, AA = amino acid, GSH, GSSG = glutathion, diglutathion, Cys = cysteine; further details are given in the text.

possibility that expression of BAT is associated with an increase in both, Na^+-dependent and Na^+-independent transport of cationic and neutral AAs across the brush-border membrane (Van Winkle, 2001). Schröder *et al.* (2003) investigating adaptive responses of intestinal AA transport in lambs and goats to a long-term low dietary protein supply reported a decreased transport capacity for the dispensable AA L-alanine whereas the transport capacity for the indispensable AA L-leucine was significantly enhanced.

Absorption of tri- and dipeptides

Until the 1970s, it was thought that the absorption of AAs by the mammalian gastrointestinal tract occurs solely in the form of free AAs after luminal hydrolysis of proteins and peptides to their monomeric constituents (Matthews and Adibi, 1976). In the meantime, most nutritionists agree that in monogastric species, the majority (> 50%) of AAs absorbed are taken up from the gastrointestinal tract as tri- and dipeptides (Leibach and Ganapathy, 1996). Calculations of net fluxes of AA nitrogen in ruminant animals across the portal-drained and mesenteric-drained viscera indicate a similar situation in ruminant species (Krehbiel and Matthews, 2003). However, somewhat different from monogastric species, where only a small portion of those peptides taken up by the enterocytes finally appears in intact form in the circulation due to extensive intracellular hydrolysis by cytosolic peptidases (Daniel *et al*., 1994; Krehbiel and Matthews, 2003), substantial amounts of small peptides deriving from intestinal absorption have been reported to occur in the portal and mesenteric vein of ruminant species. Peptide-bound AAs accounted for a higher portion of plasma AAs than represented by the fraction of free plasma AAs (Webb *et al*., 1993). On the other hand, Backwell *et al.* (1997) found no evidence for a substantial absorption of intact peptides into the circulation of sheep fed a forage-based diet. The reasons for these discrepancies with respect to the absorption of intact small peptides from the gastrointestinal tract, either between monogastrics and ruminants or between different studies in ruminants, might have several reasons, including methodological problems in plasma peptide analysis, species differences as well as influences of diet and physiological state of the animals (Backwell *et al*., 1997; Krehbiel and Matthews, 2003).

The transport protein responsible for the gastrointestinal absorption of tri- and dipeptides, named PEPT1, has been cloned from several tissues in various mammalian and non-mammalian species. The structure and function of eukaryotic peptide transporters, all belonging to the superfamily of proton oligopeptide transporters (POT), was recently reviewed by Meredith and Boyd (2000). In monogastric species, PEPT1 expression in the gastrointestinal tract appears to be restricted to the small intestine, with lower abundance in the ileum and at a very low level in the colon; no expression was found in the stomach and caecum (Meredith and Boyd, 2000). At the cellular level, PEPT1 expression in the intestine is restricted to mature enterocytes whereby expression appears to be associated with the migration of enterocytes out of the crypts onto the villus (Meredith and Boyd, 2000). In sheep and cattle, a mRNA encoding for a peptide transporter very similar to PEPT1 in humans, rats and rabbits was detected at various sites of the gastrointestinal tract. The level of expression was found to be highest in the small intestine, moderate in the omasum and low or absent in the rumen (Chen *et al*., 1999; Pan *et al*., 2001).

Although uptake of small peptides from the gastrointestinal contents has been established unequivocally using a variety of intestinal tissue preparations since more than 30-40 years, investigations of intestinal peptide transport using isolated brush-border membrane vesicles launched a new era in the field of peptide transport. This experimental approach made it possible to delineate the functional and energetic aspects of the peptide transport process in the absence of potentially complicating factors such as intracellular hydrolysis and metabolism (Ganapathy *et al*., 2001). One distinguishing feature of intestinal peptide transport is its energization by a transmembrane H^+ gradient rather than a transmembrane Na^+ gradient, which in turn

is generated by Na^+/H^+ exchange at the brush-border membrane (Figure 2), maintaining an acidic microclimate adjacent to the epithelial surface (Ganapathy *et al*., 2001). The maximum of dipeptide transport was observed in the presence of a trans-apical pH gradient and extracellular Na^+ (Thwaites *et al*., 2002). Moreover, the same authors reported that protein kinase A-mediated modulation of intestinal dipeptide absorption is indirect via effects on the apical Na^+/H^+ exchanger NHE3 (Thwaites *et al*., 2002). Following uptake of peptides into the cell, they will be largely hydrolysed by cytosolic peptidases and subsequently absorbed as free AA by AA carriers present in the basolateral membrane of enterocytes (Daniel *et al*., 1994). A small portion of peptides entering the cell via PEPT1 may although be released into the circulation by a facilitated peptide

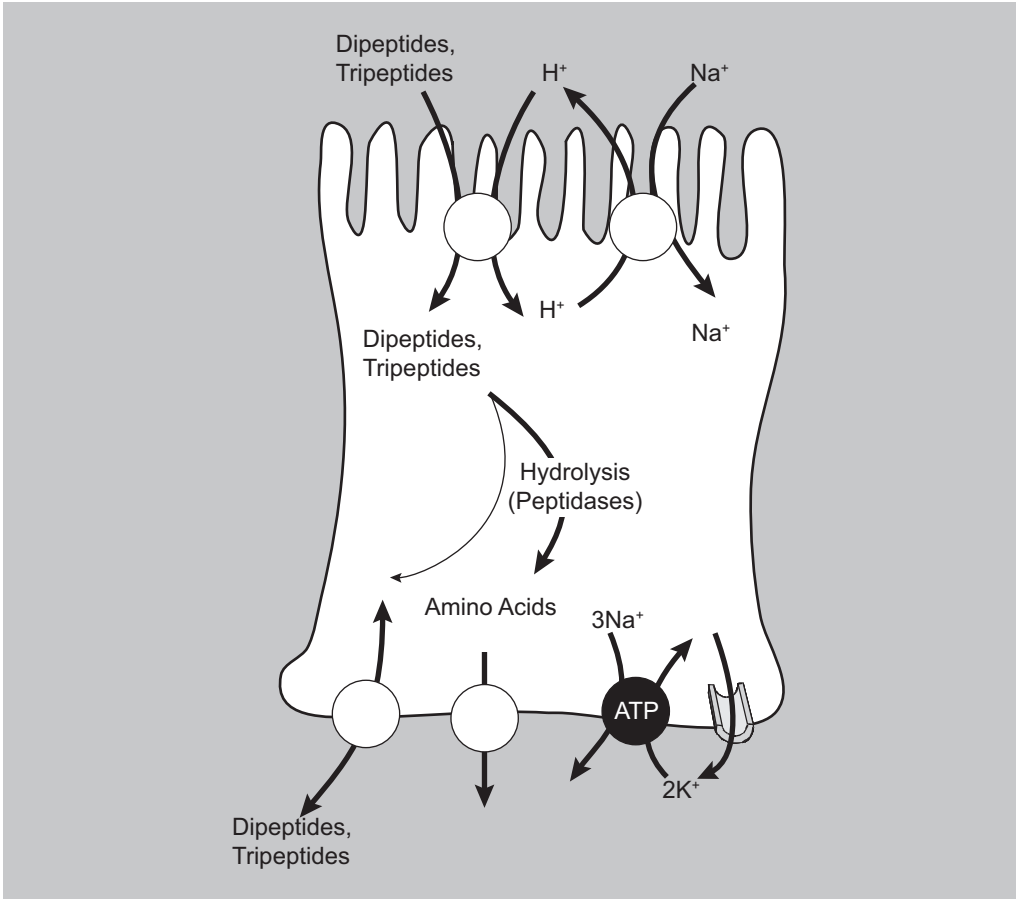

Figure 2. Model for the absorption of tri- and dipeptides from the small intestine.
Uptake is initiated by H^+-coupled transport of peptides across the brush-border membrane with the H^+-gradient being generated by Na^+/H^+-exchange at the brush-border membrane. Most peptides are then hydrolysed in the cytosol and released by AA carriers across the basolateral membrane. A minor portion of peptides may also leave the cell across the basolateral membrane by a peptide carrier distinct from that present in the apical membrane of enterocytes. For further details see text.

carrier in the basolateral membrane (Figure 2). Although this carrier is not well characterized until now, it appears that the peptide transporter in the basolateral membrane of enterocytes is clearly distinct from that expressed at the brush-border membrane (Terada *et al*., 1999).

The intestinal peptide transport system PEPT1 accepts a wide variety of chemically and structurally diverse dipeptides and tripeptides as substrates. Free AAs are not recognized as transport substrates. The carrier is stereoselective discriminating against peptides containing D- instead of L-AAs. In addition to peptides derived from protein digestion, the intestinal peptide transport system PEPT1 interacts with a variety of so-called peptidomimetics, including pharmacological relevant substances like β-lactam antibiotics, bestatin and angiotensin-converting enzyme (ACE) inhibitors (Adibi, 1997; Ganapathy *et al*., 2001).

From a functional point of view, there is strong evidence for a role of PEPT1 in the absorption of dipeptides and tripeptides from the small intestine of ruminants (Wolffram *et al*., 1998). Experimental evidence for active carrier-mediated uptake of small peptides from the forestomachs is absent (Martens *et al*., 2001), despite the fact that several studies have calculated a considerable net absorption of peptides from the forestomachs (see above). Flux rates of peptides from the luminal to the serosal site of ruminal and omasal epithelia were found to be linear over a wide concentration range of transport substrates, indicative for a diffusional rather than a carrier-mediated mechanism (Matthews and Webb, 1995; McCollum and Webb, 1998; Martens *et al*., 2001). Furthermore, a significant in vivo role of peptide absorption from the forestomachs by PEPT1 can be questioned due to the finding that short fatty acids, occurring at high concentrations within the forestomachs, significantly inhibit carrier-mediated peptide absorption in rat distal small intestine by attenuating the driving force for peptide uptake across the brush-border membrane, namely the inwardly directed H^+ gradient (Scharrer *et al*., 1999). Interestingly, a strong inhibitory influence of a mixture of short chain fatty acids on in vitro uptake of the dipeptide glycyl-sarcosine into omasal tissue preparations was found in another study. The authors speculated that short chain fatty acids may serve to attenuate paracellular transport, whereas amino acids and peptides stimulate the process (McCollum and Webb, 1998).

A functional interaction between uptake of free AAs and peptides at the cellular level is indicated by the findings of Wenzel *et al*. (2001), who reported a significant stimulation of the uptake of the cationic AA L-arginine into Caco-2 cells after pre-incuabtion of the cells with several dipeptides transported by PEPT1. Furthermore, the application of dipeptides during pre-incubation was always found to be more efficient than the application of equimolar amounts of the constituent AAs. Thus, uptake of peptides via PEPT1 with subsequent intracellular hydrolysis may augment the intracellular pool of AAs, including neutral AAs which in turn stimulates uptake of cationic and neutral AAs via system $b^{0,+}$.

Intestinal phosphate (P_i) absorption

Isolated rumen epithelial tissues from sheep have been used to clarify the potential role of the forestomach region for overall P_i absorption. Under short circuit current conditions no significant net flux rates could be determined which proved the absence of an active transport system. Since the unidirectional P_i flux rates could either be increased or decreased by setting the serosal

side to a positive (+25 mV) or negative (-25 mV) potential, it was concluded that P_i is at least partly transported in the ionized form. For the in vivo situation it is postulated that the existing electrochemical gradient may act as the driving force for passive Pi absorption across the rumen wall (Breves *et al*., 1988).

In ruminants as in monogastric species, the small intestines are the major site for P_i absorption (Pfeffer *et al*., 1970). The involvement of carrier-mediated processes had already been postulated from perfusion experiments, however, it took until the early 90s that the epithelial mechanisms were identified. From P_i-uptake studies into brush border membrane vesicles prepared from the proximal intestinal tract of sheep Shirazi-Beechey *et al*. (1991a) have postulated a H^+/ P_i cotransport which was stimulated by low P intake. This could not be confirmed by studies on jejunal unidirectional P_i flux rates in Ussing chambers using intestinal tissues from sheep and goats which have clearly shown that a substantial part of active P_i transport could be inhibited by luminal addition of arsenate, low luminal sodium concentrations and by serosal addition of ouabain. This indicates that a Na^+/ P_i cotransport is involved in intestinal P_i absorption similarly as it had already been shown for non-ruminant species. Na^+/ P_i cotransport could be upregulated by P depletion which was not associated with any changes in the Vitamin D hormone system (Schröder *et al*., 1995). Recently, this controversy could be clarified by studies on the molecular structure of intestinal P_i transporters in goats which have led to the following results: The duodenum of goats is equipped with a H^+-dependent P_i transport system which can be stimulated by increasing luminal Na^+ concentrations but which does not respond to dietary P depletion. In the jejunum a secondary active Na^+-dependent system is expressed which showed a high homology to the murine P_i transporter type NaPi IIb. This system can be upregulated by dietary P restriction (Huber *et al*., 2002). Although the P_i transport capacity was already high at birth, there seemed to be a change in the type of Na^+-dependent P_i transporter within the first weeks of life and NaPi IIb expression increased up to the fifth month of life (Huber *et al*., 2003). The present state of identified P_i-transporters which are involved in endogenous P_i circulation is shown in Figure 3.

The potential function of ileal P_i transport is not clear. From flux measurements in Ussing chambers, net P_i flux rates were calculated which were significantly higher than in any other segment of the intestinal tract (Breves, unpublished). However, with regard to pH and P_i concentrations in ileal fluid under in-vivo conditions, the biological relevance has to be questioned.

Concluding remarks

Substantial progress has been made to identify epithelial mechanisms for intestinal absorption of monosaccharides, amino acids, peptides and P_i. For P_i the most relevant Na^+ coupled system is located in the jejunum and has a high homology to the murine NaPi IIb. The physiological relevance of the H^+-dependent duodenal P_i transport system has still to be clarified. The quantity of data from ruminants on transport of amino acids and peptides is not abundant; most concepts have been transferred from monogastric species and from laboratory animals. A major topic for further research will be the quantitative contribution of peptide transport. The classical concepts of intestinal carbohydrate digestion have been changed substantially, i.e., the small intestines

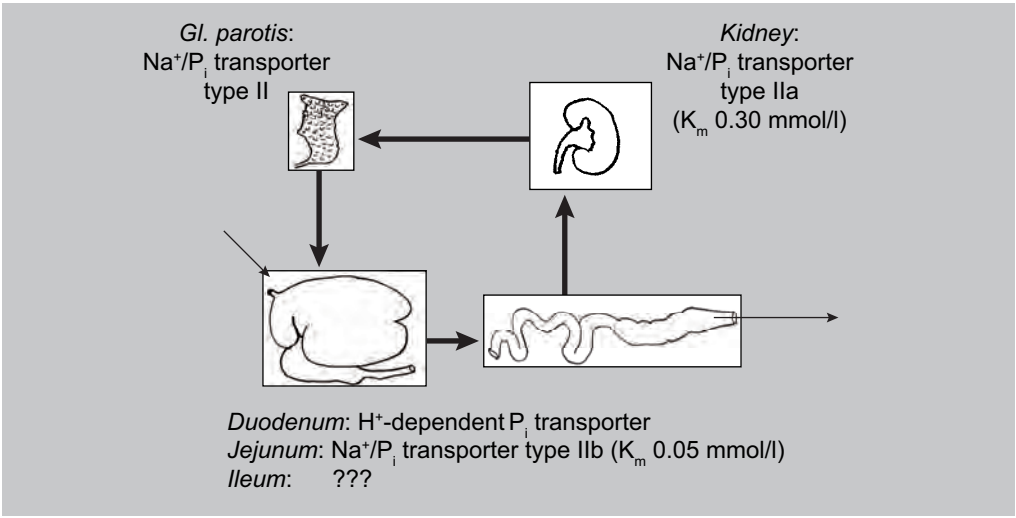

Figure 3. Identified epithelial Pi transporters in ruminants (Source: Huber et al*., 2003).*

may have a significant contribution to overall carbohydrate digestion. The potential limitations as enzymatic hydrolysis or epithelial transport have to be studied in further experiments.

As already shown for monogastric species the transport systems reviewed in this paper are characterized by common features regarding the transcellular pathway as the major absorptive route and the direct or indirect Na^+-dependency with some exceptions.

References

Adibi, S.A. (1997). The oligopeptide transporter (Pept-1) in human intestine: biology and function. Gastroenterology, 113, 332-340.

Backwell, F.R.C., M. Hipolito-Reis, D. Wilson, L.A. Bruce, V. Buchan & J.C. MacRae (1997). Quantification of circulating peptides and assessment of peptide uptake across the gastrointestinal tract of sheep. Journal of Animal Science, 75, 3315-3322.

Barker, G.A. & J.C. Ellory (1990). The identification of neutral amino acid transport systems. Experimental Physiology, 75, 3-26.

Bassett, J.M. (1975). Dietary and gastrointestinal control of hormones regulating carbohydrate metabolism in ruminants. In: I.W. McDonald & A.C.I. Warner (eds.) Digestion and Metabolism in the Ruminant. University of New England Publishing Unit, Armidale, NSW Australia, 383-398pp.

Binder, H.J. (1970). Amino acid absorption in the mammalian colon. Biochimica and Biophysica Acta, 219, 503-506.

Bird, A.R., W.J. Croom Jr., Y.K. Fan, B.L. Black, B.W. McBride & I.L. Taylor (1996). Peptide regulation of intestinal glucose absorption. Journal of Animal Science, 74, 2523-2540.

Breves, G., H. Höller, P. Packheiser, G. Gäbel & H. Martens (1988). Flux of inorganic phosphate across the sheep rumen wall in vivo and in vitro. Quarterly Journal of Experimental Physiology, 73, 343-351.

Chillaron, J., R. Estevez,, C. Mora, C.A. Wagner, H. Suessbrich, F. Lang, J.L. Gelpi, X. Testar, A.E. Busch, A. Zorzano & M. Palacin (1996). Obligarory amino acid exchange via systems $b^{0,+}$-like and y^+-like. Journal of Biological Chemistry, 271, 17761-17770.

Chen, H., E.A. Wong & K.E. Webb, Jr. (1999). Tissue distribution of a peptide transporter mRNA in sheep, dairy cows, pigs, and chicken. Journal of Animal Science, 77, 1277-1283.

Daniel, H., M. Boll & U. Wenzel, (1994). Physiological importance and characteristics of peptide transport in intestinal epithelial cells. In: Souffrant, W.-B. & H. Hagemeister, (eds.). Proceedings of the VIth International Symposium on Digestive Physiology in Pigs. Volume II, EAAP-Publication No 80, Eur. Assoc. Anim. Prod., pp. 1-7, Dummerstorf, Germany

Dyer, J., S. Vayro, T.P. King & S.P. Shirazi-Beechey (2003). Glucose sensing in the intestinal epithelium. European Journal of Biochemistry, 270, 3377-3388.

Ganapathy, V., M.E. Ganapathy & F.H. Leibach (2001). Intestinal transport of peptides and amino acids. In: Barrett, K.E. & Donowitz, M. (eds.). Gastrointestinal transport: Molecular physiology. Academic Press. Series title: Current Topics in Membranes 50, 379-412.

Harmon, D.L. (1992). Dietary influences on carbohydrases and small intestinal starch hydrolysis capacity in ruminants. Journal of Nutrition, 122, 203-210.

Hediger, M.A. & D.B. Rhoads (1994) Molecular Physiology of sodium-glucose cotransporters. Physiological Reviews, 74, 993-1026.

Helliwell, P.A. & G.L. Kellett (2002). The active and passive components of glucose absorption in rat jejunum under low and high perfusion stress. Journal of Physiology, 544, 579-589.

Huber, K., C. Walter, B. Schröder & G. Breves (2002). Phosphate transport in the duodenum and jejunum of goats and its adaptation by dietary phosphate and calcium. American Journal of Physiology, Regulatory Integrative Comparative Physiology, 283, R296-R302.

Huber, K., U. Roesler, A. Muscher, K. Hansen, I. Widiyono, E. Pfeffer & G. Breves (2003). Ontogenesis of epithelial phosphate transport systems in goats. American Journal of Physiology, Regulatory Integrative Comparative Physiology, 284, R413-R421.

Huntington, G.B. (1997). Starch utilization by ruminants: From basics to the bunk. Journal of Animal Science, 75, 852-867.

Krehbiel, C.R. & J.C. Matthews (2003). Absorption of amino acids and peptides. In: J.P.F. D'Mello (ed.). Amino acids in animal nutrition, sec. ed., CABI Publishing, pp. 41-70.

Kreikemeier, K.K., D.L. Harmon, J.P. Peters, K.L. Gross, C.K. Armendariz & C.R. Krehbiel (1990). Influence of dietary forage and feed intake on carbohydrase activities and small intestinal morphology in calves. Journal of Animal Science, 68, 2916-2929.

Leibach, F.H. & V. Ganapathy (1996). Peptide transporters in the intestine and the kidney. Annual Review in Nutrition, 16, 99-119.

Lescale-Matys, L., J. Dyer, D. Scott, T.C. Freeman, E.M. Wright & S.P. Shirazi-Beechey (1993). Regulation of the ovine intestinal Na^+-glucose co-transporter (SGLT1) is dissociated from mRNA abundance. The Biochemical Journal, 291, 435-440.

Mabjeesh, S.J., D. Guy & D. Sklan (2003). Na^+/glucose co-transporter abundance and activity in the small intestine of lambs: enhancement by abomasal infusion of casein. British Journal of Nutrition, 89, 573-580.

Mailliard, M.E., B.R. Stevens & G.E. Mann (1995). Amino acid transport by small intestinal, hepatic, and pancreatic epithelia. Gastroenterology, 108, 888-910.

Martens, H., J. Kudritzki, K. Wolf & M. Schweigel (2001). No evidence for active peptide transport in forestomach epithelia of sheep. Journal of Animal Physiology and Animal Nutrition, 85, 314-324.

Matthews, D.M. & S.A. Adibi (1976). Peptide absorption. Gastroenterology, 71, 151-161.

Matthews, J.C.(2000). Amino acid and peptide transport systems. In: J.P.F. D'Mello (ed). Farm animal metabolism and nutrition, CABI Publishing, pp. 3-23.

Matthews, J.C. & K.E. Webb Jr. (1995). Absorption of L-carnosine, L-methionine, and L-methionylglycine by isolated sheep ruminal and omasal epithelial tissue. Journal of Animal Science, 73, 3464-3475.

McCarthy, R.D., T.H. Klusmeyer, J. L. Vicini, J. H. Clark & D. R. Nelson (1989). Effect of source of protein and carbohydrate on ruminal fermentation and passage of nutrients to the small intestine of lactating cows. J. Dairy Science, 72, 2002-2016.

McCollum, M.Q. & K.E. Webb Jr. (1998). Glycyl-L-sarcosine absorption across ovine omasal epithelium during coincubation with other peptide substrates and volatile fatty acids. Journal of Animal Science, 76, 2706-2711.

Meredith, D. & C.A.R. Boyd (2000). Structure and function of eukaryotic peptide transporters. Cellular and Molecular Life Sciences, 57, 754-778.

Mills, J.A.N., J. France & J. Dijkstra (1999a). A review of starch digestion in the lactating dairy cow and proposals for a mechanistic model: 1. Dietary starch characterisation and ruminal starch digestion. Journal of Animal and Feed Sciences, 8, 291-340.

Mills, J.A.N., J. France & J. Dijkstra (1999b). A review of starch digestion in the lactating dairy cow and proposals for a mechanistic model: 2. Postruminal starch digestion and small intestinal glucose absorption. Journal of Animal and Feed Sciences, 8, 451-481.

Nocek, J.E. & S. Tamminga (1991). Site of digestion of starch in the gastrointestinal tract of dairy cows and its effect on milk yield and composition. Journal of Dairy Science, 74, 3598-3629.

Owens, F.N. , R.A. Zinn, Y.K. Kinn (1986). Limits to starch digestion in the ruminant small intestines. Journal of Animal Science, 63, 1634-1648.

Pan, Y., E.A. Wong, J.R. Bloomquist & K.E. Webb, Jr. (2001). Expression of a cloned ovine gastrointestinal peptide transporter (oPepT1) in Xenopus oocytes induces uptake of oligopeptides in vitro. Journal of Nutrition, 131, 1264-1270.

Pappenheimer, J.R. & K.Z. Reiss (1987). Contribution of solvent drag through intercellular junctions to absorption of nutrients by the small intestine of the rat. Journal of Membrane Biology, 100, 123-136.

Pfeffer, E., A. Thompson & D.G. Armstrong (1970). Studies on intestinal digestion in the sheep. 3. Net movement of certain inorganic elements in the digestive tract of rations containing different proportions of hay and rolled barley. British Journal of Nutrition, 24, 197-204.

Rechkemmer, G., G. Gäbel, L. Diernaes, J. Sehested, P. D. Møller & W. v. Engelhardt (1995). Transport of short chain fatty acids in the forestomach and hindgut. In: Engelhardt, W. v., S. Leonhard-Marek, G. Breves & D. Giesecke (eds). Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. Ferdinand Enke Verlag Stuttgart, pp. 95-116.

Reynolds, C.K., J.D. Sutton, D.E. Beever (1997). Effects of feeding starch to dairy cattle on nutrient availability and production. In: P. C. Garnsworth, J. Wiseman & W. Haresign (eds.). Recent advances in animal nutrition. Nottingham University Press, Nottingham, pp. 105-133.

Russell, J.R., A.W. Young & N.A. Jorgensen (1981). Effect of dietary corn starch intake on pancreatic amylase and intestinal maltase and pH in cattle. Journal of Animal Science, 52, 1177-1182.

Scharrer, E. (1976). Developmental changes of sugar transport in the ovine small intestine. Pflügers Archiv, 366, 147-151.

Scharrer, E., I. Amstutz & B. Grenacher (1999). Inhibition of mucosal glycylsarcosine uptake by acetate in rat distal small intestine. Experimental Physiology, 84, 541-548.

Scharrer, E., H.-G. Liebich, W. Raab & N. Promberger (1979). Influence of age and rumen development on intestinal absorption of galactose and glucose in lambs. Zentralblatt für Veterinärmedizin Reihe A, 26, 95-105.

Schröder, B., H. Käppner, K. Failing, E. Pfeffer & G. Breves (1995). Mechanisms of intestinal phosphate transport in ruminants. British Journal of Nutrition, 74, 635-648.

Schröder, B., M. Schöneberger M. Rodehutscord, E. Pfeffer & G. Breves (2003). Dietary protein reduction in sheep and goats: different effects on L-alanine and L-leucine transport across the brush-border membrane of jejunal enterocytes. Journal of Comparative Physiology, B 173, 511-518.

Shirazi-Beechey, S.P., R.B. Beechey, J. Penny, S. Vayro, W. Buchan & D. Scott (1991a). Mechanisms of phosphate transport in sheep intestine and parotid gland: response to variation in dietary phosphate supply. Experimental Physiology, 76, 231-241.

Shirazi-Beechey, S.P., B.A. Hirayama, Y. Wang, D. Scott, M.W. Smith & E.M. Wright (1991b). Ontogenic development of lamb intestinal sodium-glucose cotransporter is regulated by diet. Journal of Physiology, 437, 699-708.

Shirazi-Beechey, S.P., I.S. Wood, J. Dyer, D. Scott & T.P. King (1995). Intestinal sugar transport in ruminants. In: Engelhardt, W. v., S. Leonhard-Marek, G. Breves & D. Giesecke (eds). Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. Ferdinand Enke Verlag Stuttgart, pp. 117-133.

Stevens, B.R., J.D. Kaunitz & E.M. Wright (1984). Intestinal transport of amino acids and sugars: advances using membrane vesicles. Annual Review of Physiology, 46, 417-433.

Terada, T., K. Sawada, H. Saito, Y. Hashimoto & K.-L. Inui, (1999). Functional characteristics of basolateral peptide transporter in the human intestinal cell line Caco-2. American Journal of Physiology, 276, G1435-G1441.

Thorens, B. (1996). Glucose transporters in the regulation of intestinal, renal and liver glucose fluxes. American Journal of Physiology (Gastrointest. Liver Physiol. 33), 270, G541-G553.

Thwaites, D.T., D.J. Kennedy, D. Raldua, C.M. Anderson, M.E. Mendoza, C.L. Bladen & N.L. Simmons (2002). H^+/dipeptide absorption across the human intestinal epithelium is controlled indirectly via a functional Na^+/H^+ exchanger. Gastroenterology, 122, 1322-1333.

Van Winkle, L.J. (2001). Genetic regulation of expression of intestinal biomembrane transport proteins in response to dietary protein, carbohydrate, and lipid. Molecular physiology. In: Barrett, K.E. & M. Donowitz (Eds.). Academic Press. Series title: Current Topics in Membranes, 50, 113-161.

Walker, J.A. & D.L. Harmon (1995). Influence of ruminal or abomasal starch hydrolysate infusion on pancreatic exocrine secretion and blood glucose and insulin concentrations in steers. Journal of Animal Science, 73, 3766-3774.

Webb, K.E., Jr., D.B. Direienzo & J.C. Matthews (1993). Recent developments in gastrointestinal absorption and tissue utilization of peptides: a review. Journal of Dairy Science, 76, 351-361.

Wenzel, U., B. Meissner, F. Döring & H. Daniel (2001). PEPT1-mediated uptake of dipeptides enhances the intestinal absorption of amino acids via transport system $b^{0,+}$. Journal of Cell Physiology, 186, 251-259.

Wolffram, S., B. Grenacher & S. Scharrer (1998). H^+-coupled uphill transport of the dipeptide glycylsarcosine by bovine intestinal brush-border membrane vesicles. Journal of Dairy Science, 81, 2595-2603.

Wood, I.S., J. Dyer, R.R. Hofmann & S.P. Shirazi-Beechey (2000). Expression of the Na^+/glucose co-transporter (SGLT1) in the intestine of domestic and wild ruminants. Pflügers Archiv – European Journal of Physiology, 441, 155-162.

Zhao, F.-Q., E.K. Okine, C.I. Cheeseman, S.P. Shirazi-Beechey & J.J. Kennelly (1998). Glucose transporter gene expression in lactating bovine gastrointestinal tract. Journal of Animal Science, 76, 2921-2929.

Urea transporters and urea recycling in ruminants

J.C. Marini[1], J.M. Sands[2] and M.E. Van Amburgh[3]
[1]Department of Animal Science, University of Illinois, Champaign-Urbana, IL 6180, USA
[2]Renal Division, Department of Medicine, and Department of Physiology, 1639 Pierce Drive, WMB 338, Emory University School of Medicine, Atlanta, GA 30322, USA
[3]Department of Animal Science, 272 Morrison Hall, Cornell University, Ithaca, NY, 14853, USA

Abstract

Urea is considered an end product of nitrogen metabolism in many species; however, it plays a central role in the nitrogen economy of ruminants. Ruminant animals are able to recycle substantial amounts of urea into the gastrointestinal tract rather than excreting it in the urine. After hydrolysis to ammonia, gut bacteria can use the nitrogen from urea to satisfy their metabolic needs ultimately providing the host ruminant animal with amino acids, nucleic acids and ammonia.

Since this "protein regeneration cycle" (Houpt, 1959) was postulated almost 50 years ago, the quantitative response in urea kinetics resulting from low protein diets, pregnancy, lactation, growth or thirst has been documented. Although, the adaptive physiological response of the kidney in the conservation of urea to these different situations is well established, the precise salvage mechanisms of urea and its transfer into the gastrointestinal tract have remained elusive.

The cloning and characterization of urea transporters in the kidney, followed by their identification in gut tissue, and more recently in the rumen of cattle and sheep, provide a putative mechanism with the potential for a coordinated response to a variety of physiological conditions. More research is needed to fully integrate molecular aspects of urea metabolism with the whole animal urea kinetics. A better understanding of the capability of ruminants to recycle urea is needed to maximize nitrogen usage in modern feeding conditions, minimize the feeding of nitrogen, and decrease the amount of nitrogen excreted into the environment.

Keywords: Nitrogen metabolism, nitrogen recycling, urea kinetics, urea transporter

Introduction

Urea is often considered a waste product of protein metabolism, resulting from the need to detoxify ammonia arising from the oxidation of amino acids. However, urea has many other functions in different organisms. For example, in the kidney urea is essential for concentrating urine (Sands *et al*., 1992), in marine animals it has an important osmotic role (Goldstein, 1970), in snails it is involved in shell formation (Campbell, 1970) and in ruminants urea is part of the "protein regeneration cycle" (Houpt, 1959). Urea is a small (MW 60) molecule that was thought to diffuse freely across cell membranes. Urea permeability across artificial lipid bilayers, however, is quite low (4×10^{-6} cm/s), consistent with urea being highly polar. Nonetheless, a 100 to 1000-fold increase was observed when urea permeability was measured in cell membranes rather than in

artificial lipid vesicles (Gallucci *et al*., 1975). Some species, notably elasmobranch fishes (Barton *et al*., 1999) and sharks (Wells *et al*., 1986) are able to maintain urea concentrations greater than 300 mM in an aquatic environment. It seems obvious that, at least in these species, a tight system for urea conservation is present.

Ruminant animals, in which hepatic synthesis of urea exceeds apparent digestible nitrogen intake, seem to also fall within this category. Without urea recycling, negative nitrogen balances would be observed even when fed high protein diets. Quantitative aspects of urea recycling are discussed in a recent review by Lobley and Lapierre (2001).

Urea excretion and its regulation by the kidney

Bodil Schmidt-Nielsen and coworkers (Schmidt-Nielsen and Osaki, 1958; Schmidt-Nielsen *et al*., 1958) were the first to suggest that the kidney was involved in the reabsorption of urea in sheep and that the animals were able to increase urea reabsorption when fed low protein diets.

Changes in renal hemodynamic parameters in response to feeding or protein content of the diet are well established. Circadian changes in renal blood flow have been observed in sheep with minimal values immediately before feeding (Denis *et al*., 2004). Changes in renal plasma flow and glomerular filtration rate (GFR) have been reported in animals fed low protein diets (Eriksson and Valtonen, 1982; Leng *et al*., 1985; Silanikove, 1986; Faix *et al*., 1988; Cirio *et al*., 1990; Tebot *et al*., 1998). The filtration fraction, i.e., the ratio between GFR and renal plasma flow, was also affected by low protein diets, decreasing from 25 to 15% (Ergene and Pickering, 1978). The mechanism of action of protein nutrition on these variables is not well understood, but several metabolites and hormones (IGF-1, glucagon, plasma amino acid concentration, arginine, NO) related to amino acid availability have been proposed (el Sayed *et al*., 1991; Leng *et al*., 1994; Lang *et al*., 1995; Bachmann and Oberbaumer, 1998; Wilcox and Welch, 1998). These hemodynamic changes are geared to reduce the tubular load of urea and thus its excretion. The effect of protein nutrition on GFR is not limited to ruminants; GFR was shown to increase in dogs and humans after a protein meal (O'Connor and Summerill, 1976; King and Levey, 1993, respectively) or after an infusion of some individual amino acids (Lee and Summerill, 1982). Urea seems not to affect GFR directly. The intravenous infusion of urea in sheep fed low protein diets took 19 h to restore the GFR to levels seen in animals on a normal protein diet (Ergene and Pickering, 1978). This suggests that urea was recycled into the rumen, where it was converted into microbial protein, and then made available to the animal. It was concluded that the change in amino acid availability, rather than urea per se, mediated the change in GFR.

In kidney, urea transporter (UT) proteins mediate the transport of urea across the inner medullary collecting ducts (IMCD; reviewed in Bagnasco (2003), Sands (2003a, 2003b, 2004), Shayakul and Hediger (2004)). Urea is thus not only salvaged from excretion, but is also used to maintain the osmotic gradient required to concentrate urine.

Although the initial evidence for the existence of kidney UT came from functional measurements of urea permeability in the perfused terminal IMCD of rats (Sands *et al*., 1987), it wasn't until the early 90's that UT were characterized. The first transporter cloned was UT-A from rabbit renal

medulla (You *et al*., 1993), followed by UT-B from a human erythroleukemic cell line (Olives *et al*., 1994). Both transporters belong to the same solute carrier family (*Scl14*), but while the *Scl14A2* (UT-A) gene encodes for 6 proteins and 9 cDNA isoforms, the *Scl14A1* (UT-B) gene encodes for a single isoform (Table 1). The *Scl14A2* gene is also atypical, having two promoter elements: promoter I in the typical location upstream of exon 1 which drives transcription of UT-A1, UT-A1b, UT-A3, UT-A3b, and UT-A4; and promoter II located within intron 12 which drives transcription of UT-A2 and UT-A2b (Bagnasco *et al*., 2000; Nakayama *et al*., 2001).

Phylogenetic analysis indicates that all urea transporters arose from a primordial urea transporter that underwent two gene duplication events during evolution (Minocha *et al*., 2003). For example, there is a 60% similarity between human UT-A and UT-B sequences.

UT-B has been cloned in humans, rats and mice (Couriaud *et al*., 1996; Sands *et al*., 1997; Tsukaguchi *et al*., 1997; Yang *et al*., 2002), and more recently in cattle (Stewart *et al*., 2004). The amino acid sequence of bovine UT-B shared 85 and 80% identity with mouse and human, respectively. Although UT-A has been cloned in humans, rats and mice (Nakayama *et al*., 2001; Bagnasco *et al*., 2001; Fenton *et al*., 2002a), only a 770 bp fragment encoding 257 amino acids has been cloned in sheep (Artagaveytian *et al*., 2005). The deduced amino acid sequence of sheep UT-A shared 92, 93 and 92% identity with rabbit, human and rat, respectively (Artagaveytian *et al*., 2005).

Most UT-A isoforms are expressed in the kidney (Figure 1). Both UT-A1 and UT-A3 occur in IMCD cells (Nielsen *et al*., 1996; Karakashian *et al*., 1999; Bagnasco *et al*., 2001; Kim *et al*., 2002).

In rats, UT-A1 and UT-A3 are expressed in the apical membrane, while in mice, UT-A1 is apical but UT-A3 is basolateral (Nielsen *et al*., 1996; Bagnasco *et al*., 2001; Terris *et al*., 2001; Kim *et al*., 2002; Stewart *et al*., 2004b). The reason for this species difference is still unknown. UT-A2 is found in thin descending limbs (You *et al*., 1993; Nielsen *et al*., 1996; Wade *et al*., 2000; Kim *et al*., 2002), whereas UT-A4 is expressed in rat kidney medulla (but not in mouse), although the exact location of UT-A4 is not certain (Fenton *et al*., 2002a, 2002b; Klein *et al*., 2004). Additionally,

Table 1. Mammalian facilitated urea transporters.

Gene	Isoform	Localization
Slc14A1	UT-B	red blood cells, descending vasa recta, endothelial cells
Slc14A2	UT-A1/UT-A1b	inner medullary collecting duct
	UT-A2/UT-A2b	thin descending limb, liver, heart
	UT-A3/UT-A3b	inner medullary collecting duct
	UT-A4	rat medulla (not detected in mouse)
	UT-A5	testis
	UT-A6	intestine

The original references can be found in the following reviews: (Bagnasco, 2003; Kim *et al*., 2005; Sands, 2003a; 2003b; 2004; Shayakul and Hediger, 2004).

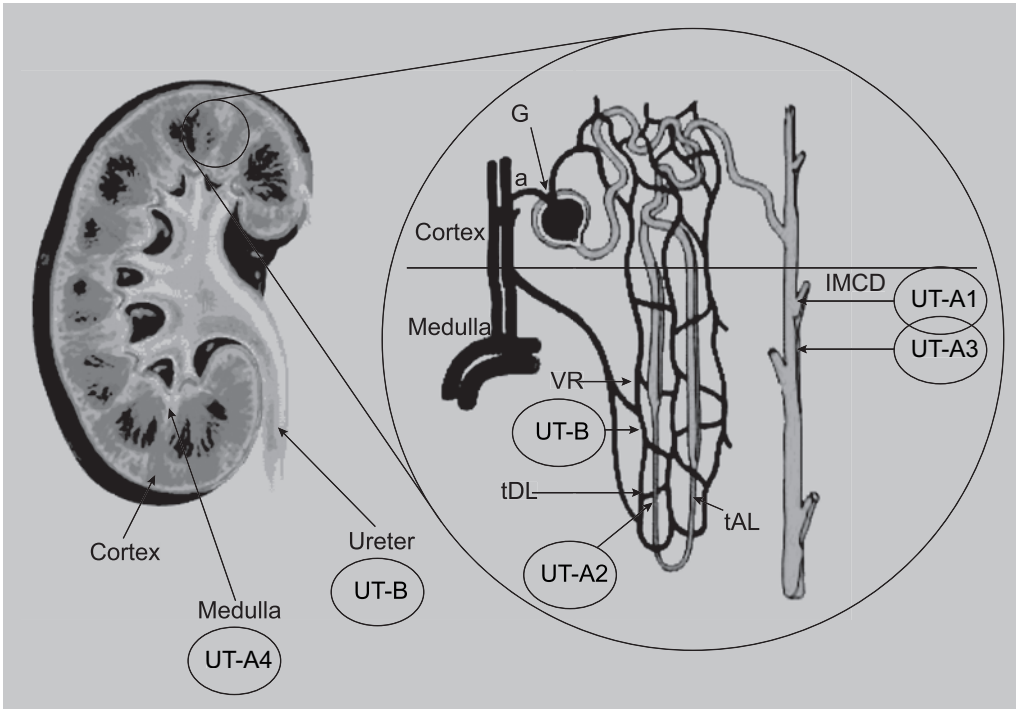

Figure 1. Schematic representation of kidney and juxtamedullary nephron showing the different location of urea transporters. UT-A1 and UT-A3 are present in the inner medullary collecting ducts (IMCD), UT-A2 in the thin descending limb (tDL), UT-A4 in the medulla, although its exact location hasn't been determined yet. UT-B is present in the vasa recta (VR) and the ureter. Also shown the glomerulus (G) and the afferent artery (a).

three UT-A cDNA variants with alternative 3'-untranslated regions have been identified, cloned and named UT-A1b, UT-A2b, and UT-A3b, respectively (Bagnasco *et al.*, 2000). Different states of glycosylation also result in different UT-A forms (Bradford *et al.*, 2001).

In humans, the UT-B protein is the erythrocyte Kidd antigen, a minor blood group antigen, and several mutations of the UT-B/Kidd antigen gene have been reported (Olives *et al.*, 1995, 1996; Lucien *et al.*, 1998, 2002; Sidoux-Walter *et al.*, 2000). UT-B protein and phloretin-inhibitable urea transport are present in rat descending vasa recta (Figure 2; Trinh-Trang-Tan *et al.*, 1987; Pallone, 1994; Pallone *et al.*, 1994, 1995; Xu *et al.*, 1997; Hu *et al.*, 2000; Timmer *et al.*, 2001; Yang *et al.*, 2002).

Originally, UT were thought to be expressed only in erythrocytes and kidney. Later, UT-A5 and UT-A6 were cloned and shown to be expressed exclusively in testis and intestine, respectively (Fenton *et al.*, 2000; Kim *et al.*, 2005). Several studies now show that both UT-A and UT-B are more widely distributed than previously thought, including brain, liver, and heart (reviewed in Bagnasco, 2003; Sands, 2003a, 2003b; Shayakul and Hediger, 2004), cochlea, ureter, and bladder (Kwun *et al.*, 2003; Spector *et al.*, 2004). It also seems that the site of expression is species specific;

for example, UT-B is expressed in the human colon while UT-A is expressed in the mouse colon (Inoue *et al*., 2004; Stewart *et al*., 2004a). We showed that UT-B is also expressed in rumen of both cattle and sheep (Marini andVan Amburgh, 2003; Marini *et al*., 2004) and UT-A in expressed in lamb liver and duodenum (Marini *et al*., 2004).

The advent of gene knock-out technology has allowed for further examination of the function of these transporters. A reduced ability to concentrate urine was observed in UT-A1/UT-A3 knock out mice on a normal protein diet, together with a reduced inner medullary interstitial urea content, and a lack of vasopressin-stimulated or phloretin-inhibitable urea transport in their IMCD (Fenton *et al*., 2004). However, UT-B knock-out animals have only a mildly reduced urine concentrating ability (Yang *et al*., 2002), similar to the phenotype in people who lack UT-B/Kidd antigen (Sands *et al*., 1992). In contrast to wild-type mice, urea loading does not improve urine concentrating ability in the UT-B knock-out mice (Bankir *et al*., 2004). These findings suggest that UT-B-mediated urea transport in erythrocytes and/or descending vasa recta is needed to preserve the efficiency of countercurrent exchange (Macey, 1984; Bankir *et al*., 2004). UT-B knock-out mice also have an increase in UT-A2 protein abundance, while UT-A1 and UT-A3 abundances are unchanged (Klein *et al*., 2004). Both UT-B and UT-A2 mediate urea-recycling pathways through descending vasa recta and thin descending limb, respectively (Knepper and Roch-Ramel, 1987). Thus, UT-A2 may be up-regulated to partially compensate for the loss of urea recycling through UT-B, which may explain the mild phenotype observed in UT-B knock-out mice and in people who lack UT-B/Kidd antigen.

In addition to facilitated urea transporters, two types of active urea transport are present in the rat collecting duct: sodium-urea co-transport and sodium-urea counter-transport (reviewed in Sands (2003a, 2003b)). Although no sodium-dependent active urea transporter has been cloned to date, a proton-urea co-transporter (DUR3) has been cloned from *Arabidopsis* (Klebl *et al*., 2003; Liu *et al*., 2003). In addition, some sodium-coupled co-transporters (rabbit sodium-glucose co-transporters 1 and 3, and human sodium-chloride-GABA co-transporter 1) behave as urea channels when expressed in *Xenopus* oocytes (Leung *et al*., 2000; Panayotova-Heiermann and Wright, 2001).

Thus, the discovery of UT provided the mechanism of urea reabsorption by the sheep kidney observed by Schmidt-Nielsen and coworkers (Schmidt-Nielsen and Osaki, 1958; Schmidt-Nielsen *et al*., 1958). When rats were fed low protein diets, two new UT processes were induced in the kidney (Isozaki *et al*., 1994). The first response was observed two weeks after the animals were put on a low protein diet and consisted of an ADH-dependent facilitated-diffusion urea transporter in the initial IMCD. With continued protein restriction (4 weeks), an active urea transporter was induced in the initial IMCD, contributing to the maximal decrease in fractional excretion of urea. Although to date, no differential expression of renal UT in ruminants fed low protein diets has been reported, the increase in urea reabsorption is well documented (Phillips *et al*., 1975; Eriksson and Valtonen, 1982; Leng *et al*., 1985; Faix *et al*., 1988; Cirio and Bovin, 1990; Cirio *et al*., 1990; Marini and Van Amburgh, 2003).

The increase in the salvage of urea by the kidney is not limited to low protein diets, but is also present when the requirement for N increases. Lactation has been shown to reduce kidney urea

clearance in ewes (Benlamlih *et al*., 1985; Faichney and White, 1988; Ndibualonyi *et al*., 1998) and increase urea reabsorption by the kidney in cattle (Maltz and Silanikove, 1996) and sheep (Nolan and Leng, 1970). Glomerular filtration rate increased (Benlamlih and de Pomyers, 1989) or decreased (Ndibualonyi at al, 1998) in pregnant ewes and goats, but urea excretion consistently decreased, allowing more urea to be recycled into the GI tract (Nolan and Leng, 1970; Maltz *et al*., 1981).

The role of the kidney in regulation of electrolyte and water metabolism and balance is well documented (Guyton, 1996). Dehydration increases the anti-diuretic hormone (ADH) levels, resulting in the reabsorption of free water, a decrease in urine flow and an increase in the osmolality of the urine (Yesberg *et al*., 1970). Anti-diuretic hormone is a small peptide that is synthesized in the supraoptic nuclei of the neurosecretory cells of the hypothalamus and released by the posterior pituitary when osmoreceptors are stimulated by an increase in the plasma osmotic pressure (Guyton, 1996). The role of ADH in ruminants remained controversial until Brook *et al.* (1968) and Yesberg *et al.* (1970) established that the mechanism of action was identical to that in non-ruminants. Anti-diuretic hormone could reduce the GFR by increasing the blood pressure at the afferent arteriole (Figure 1) and thus reducing the filtration pressure at Bowman's capsule, although this effect is not always present (Cross *et al*., 1966). It also acts by increasing the permeability of free water from the distal convoluted tubule and the collecting ducts of the nephron. The ADH molecules bind to V2 receptor molecules on the cell membranes of the epithelial cells of the collecting ducts. Once bound to the receptor, and by activation of the adenylyl cyclase, the cells increase the rate of cyclic AMP synthesis, which in turn activates protein kinase A. The phosphorylation of aquaporin 2 (AQP2), at serine-256, inserts AQP2 water channels into the apical plasma membrane, and increases water absorption across the collecting duct (Nielsen *et al*., 2002). In fact, the major mechanism for ADH-stimulated water absorption is the regulated trafficking of AQP2 between sub-apical vesicles and the apical plasma membrane. A similar mechanism of ADH dependent UT-A1 phosphorylation exists which increases the number of functional UT-A1 without changing the transporters' affinity (Sands, 1999). In fact, injection of ADH in sheep fed high protein diets increased the absorption of urea by the kidneys (Boldizarova *et al*., 1999).

It is not clear if urea contributes to the regulation of ADH levels and thus to the control of UT function. Research in goats has shown that urea infused into the lateral ventricle of the brain, albeit at pharmacological levels, was able to inhibit the basic ADH secretion of non-hydrated goats (Rundgren *et al*., 1979). It is not known if physiological levels of urea are able to induce the same response, although the presence of UT in brain (Ripoche and Rousselet, 1996) might be involved in this activity.

Water restriction increases ADH levels in blood (Yesberg *et al*., 1970), decreases urine flow and increases urine osmolality and increases plasma urea nitrogen (PUN) levels (Utley *et al*., 1970a; Yesberg *et al*., 1970). Depending on the severity of the restriction dry matter intake (DMI) is usually depressed (Utley *et al*., 1970a, 1970b; Mousa *et al*., 1983), although moderate restriction (80% ad libitum water intake) seems not to affect intake (Utley *et al*., 1970b).

Several authors (Cross *et al*., 1966; Utley *et al*., 1970a, 1970b; Yesberg *et al*., 1970; Khan *et al*., 1979; Bohra and Gosh, 1983) have reported a decrease in the clearance of urea by the nephron when the animals had a restricted access to water, but due to the higher PUN observed, the amount of urea excreted could remain similar (Khan *et al*., 1979; Bohra and Gosh, 1983) or decrease (Maltz *et al*., 1981; Mousa *et al*., 1983). The increase in PUN due to a decrease in urea clearance, however, did not translate into an increase in N recycling and retention because most of the diets fed in these experiments were of low digestibility and thus the demand for N very limited. In contrast, some researchers reported that, even when DMI was reduced, water restriction increased N retention in cattle (Utley *et al*., 1970a, 1970b) and goats (Mousa *et al*., 1983) fed low protein diets. The urea pool half-life increased in water-restricted animals (Mousa *et al*., 1983) suggesting that urea was being retained, but that passage to the gut remained low.

Urea entry into the gastrointestinal tract

Urea enters the gastrointestinal tract (GIT) with digestive secretions (Somers, 1961a; Nolan *et al*., 1973) and in small quantities with the bile and pancreatic juice (Varady *et al*., 1979), but the main route is through the gut wall (Houpt, 1959). Saliva plays a critical role in the digestive function of ruminants, facilitating mastication and rumination, buffering the ruminal pH and providing nutrients for the rumen microflora (Beauchemin, 1991). It also provides urea, which accounts for 50 to 80% of the total nitrogen present in saliva (Bailey and Balch, 1961a; Somers, 1961a ; Boivin and Bost, 1977) and is related to the nitrogen content of the diet (Somers, 1961b, 1961c; Marini and Van Amburgh, 2003). Variations in plasma urea nitrogen (PUN) accounted for 98% of the variation in salivary urea concentration (Harrop and Phillipson, 1974; Norton *et al*., 1982), increasing 0.65 to 0.75 units for every unit of PUN increased in the observed range of 1-20 mg dL^{-1} (Bailey and Balch, 1961b; Marini and Van Amburgh, 2003).

The entry of urea into the rumen through the epithelium was first described by Houpt (1959) in isolated rumen pouches filled with saline. Urea was hydrolyzed immediately upon entry into the pouch (Houpt, 1959; Houpt and Houpt, 1968) and the presence of carbohydrates in the rumen increased the disappearance of intravenously infused urea. Houpt and Houpt (1968) suggested that the cornified layer of the epithelium was a major barrier to urea movement. The disruption of the keratinized ruminal epithelium with NaOH caused the removal of the cornified layers, but without signs of damage to the deeper cells, and increased the rate of transfer of urea from the blood to the rumen 50 fold (Houpt and Houpt, 1968).

The intraruminal infusion of products of the fermentation of carbohydrates (CO_2, butyrate, propionate) has been related to an increase in the transfer of urea through the rumen wall. It is believed that the mechanism of action is local, increasing blood flow in the case of CO_2 (Thorlacius *et al*., 1971) or causing modification of the rumen epithelium by volatile fatty acids (Norton *et al*., 1982; Krehbiel *et al*., 1992; Sutoh *et al*., 1994). More intriguing is the increase in the urea transferred into the digestive tract when propionate was infused into the abomasum (Kim *et al*., 1999).

Although it is evident that the clearance of urea into the GIT (Figure 2 A) increases when ruminants are fed low protein diets (Kennedy and Milligan, 1980; Marini and Van Amburgh,

2003; Marini *et al*., 2004), the actual mechanism has remained elusive. It is worth noting that the changes in renal clearance of urea were small compared to the GIT clearance (Figure 2 A).

It has been suggested that PUN concentration, and thus pool size, determines the entry of urea into the GI tract (Harmeyer and Martens, 1980). Rumen ammonia concentration increased linearly in steers fed a low protein diet and infused intravenously with increasing levels of urea (Marini, 1998); this increase, however, seemed to be greater following a meal, despite constant PUN concentrations. Nonetheless, there was a limit to the amount of urea transferred when urea was infused intravenously, this occurred at PUN concentrations around 10-12 mg dL^{-1} (Vercoe, 1969; Thornton, 1970). If urea concentration were the main factor regulating the amount of urea recycled, no changes in the gastrointestinal clearance rate of urea would be expected. Our own data showed that heifers increased the GIT clearance rate of plasma urea when fed low protein diets (Figure 2); however, a similar amount of urea-N was transferred into the GIT despite wide differences in the observed PUN levels (Marini and Van Amburgh, 2003). Furthermore, a recent analysis of published data has failed to show any relationship between portal drained viscera urea transfer and urea concentration (Lobley and Lapierre, 2001).

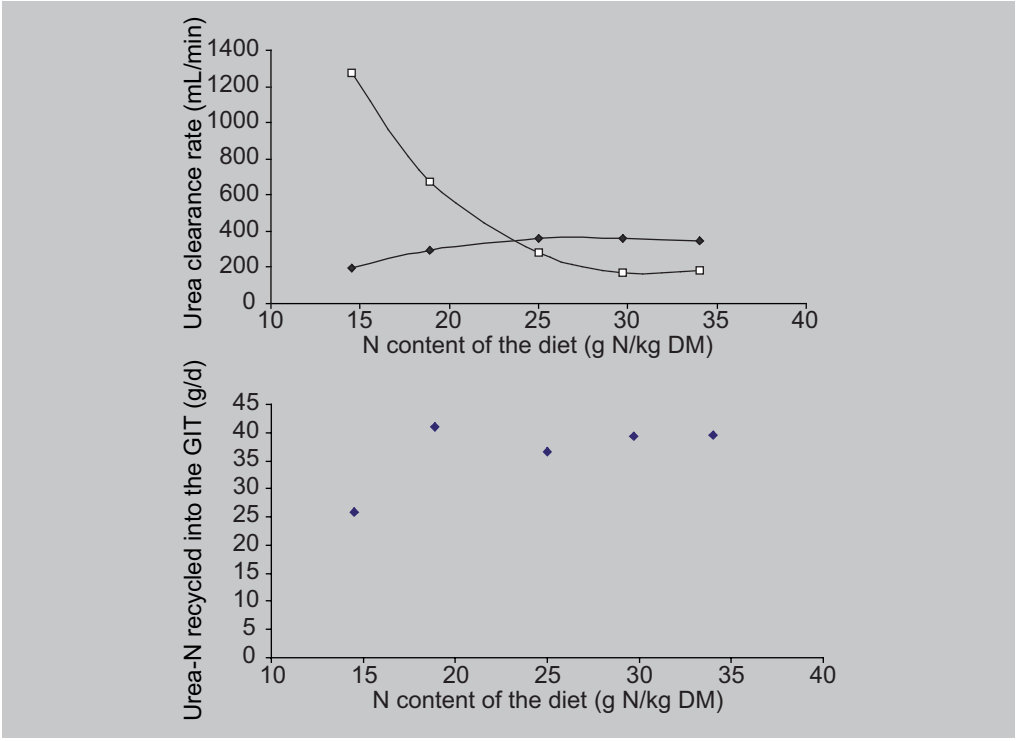

Figure 2. Panel A. Plasma renal (◆) and gastrointestinal clearance rate (□) of urea in Holstein heifers fed isocaloric diets (n= 4, SEM renal 41.0 mL/min, SEM GIT= 133.8 mL/min).
Panel B. Urea-nitrogen recycled into the gastrointestinal tract of Holstein heifers fed isocaloric diets (n= 4, SEM = 6.39 g urea-N/d).

The observation that ruminal urease activity decreases when animals were fed high protein diets prompted the proposal of urease as the mechanism controlling the passage of urea into the rumen (Cheng and Wallace, 1979; Wallace *et al*., 1979). While it is true that the hydrolysis of urea into ammonia maintains a gradient for urea transfer, inhibition of urease does not prevent the entry of urea into the rumen. Data from experiments with gnotobiotic lambs (Cheng and Wallace, 1979) and sheep infused with urease inhibitors (Whitelaw *et al*., 1991) showed that urea does enter the rumen despite the absence of urease activity and that rumen urea concentration reaches plasma values, enlarging the urea space and pool size. Furthermore, it seems that urease activity is seldom limiting (Harrop, 1974). Measured urease activity of rumen contents ranged from 80 to 100 mg urea-N dL^{-1} h^{-1} (Pearson and Smith, 1943; Bloomfield *et al*., 1960), a capacity several times higher than the amount of urea transferred into the GI tract.

As pointed out by Norton *et al.* (1982), this theory of urease activity as the mechanism controlling urea transfer across the rumen wall assumes that: 1) the urease activity of rumen wall bacteria limits the rate of urea hydrolysis; 2) liberated ammonia would exist as ammonia and not as ammonium ions and would diffuse against a concentration gradient into rumen fluid rather than down the concentration gradient back to the blood; and 3) whilst microbial urease (MW at least 130,000) can penetrate the epithelial layer, the much smaller urea molecule (MW 60) cannot diffuse through the same mucosa layer.

Active transport of urea across rumen preparations has been reported (Mooney and O'Donovan, 1970), with urea being transported from the mucosal to the serosal side against a concentration gradient when ATP was added. The identification UT mRNA in rumen and colon mucosa of sheep (Ritzhaupt *et al*., 1997; 1998) and UT in rumen and colon of sheep and cattle (Marini and Van Amburgh, 2003; Marini *et al.* 2004) provides a putative mechanism of urea transfer across the GIT epithelium. The presence in both the kidney and GIT of the same or similar transporters could thus provide the system with the potential for a coordinated response to a low protein diet (Jackson, 1998).

Future work and summary

The identification, characterization and function of urea transporters provide a novel mechanism for the understanding of urea transactions in ruminants. However, our current understanding is still limited and incomplete. Numerous possible regulation points in the expression, the differential splicing (resulting in multiple isoforms), and phosphorylation of the transporters, as well as the variations observed in different species, require further research. Whereas most of the available work on UT has been done in single cell epithelia, the multilayer keratinized ruminal epithelium offers an added level of complexity.

The integration of these molecular events with whole body urea kinetics and the known physiological responses to a variety of conditions offers an exciting challenge to ruminant physiologists.

Acknowledgements

Dr. Sands' work was supported by National Institutes of Health grants R01-DK41707 and R01-DK63657 and Dr. Marini was supported by National Institutes of Health grants R01-DK54450.

References

Artagaveytia, N., J.M. Elalouf, R. Boivin, C. de Rouffignac & A. Cirio (2005). Expression of urea transporter (UT-A) mRNA in papilla and pelvic epithelium of kidney in normal and low protein fed sheep. Comparative Biochemistry and Physiology Part B, 140, 279-285.

Bachmann, S. & I. Oberbaumer (1998). Structural and molecular dissection of the juxtaglomerular apparatus, new aspects for the role of nitric oxide. Kidney International, 67, S29-S33.

Bagnasco, S.M. (2003). Gene structure of urea transporters. American Journal of Physiology Renal Physiology, 284,F3-F10.

Bagnasco, S.M., T. Peng, M.G. Janech, A. Karakashian & J.M. Sands (2001). Cloning and characterization of the human urea transporter UT-A1 and mapping of the human *Slc14a2* gene. American Journal of Physiology Renal Physiology, 281,F400-F406.

Bagnasco, S.M., T. Peng, Y. Nakayama & J.M. Sands (2000). Differential expression ofindividual UT-A urea transporter isoforms in rat kidney. Journal of the American Society of Nephrology, 11:1980-1986.

Bailey, C.B. & C.C. Balch (1961a). Saliva secretion and its relation to feeding in cattle. 1. The composition and rate of secretion of parotid saliva in small steer. British Journal of Nutrition 15, 371-382.

Bailey, C.B. & C.C. Balch (1961b). Saliva secretion and its relation to feeding in cattle. 2. The composition and rate of secretion of mixed saliva in the cow during rest. British Journal of Nutrition 15, 83-402.

Bankir, L., K. Chen & B. Yang (2004). Lack of UT-B in vasa recta and red blood cells prevents urea-induced improvement of urinary concentrating ability. American Journal of Physiology Renal Physiology 286, F144-F151.

Barton, K.N., M.M. Buhr & J.S. Ballantyne (1999). Effects of urea and trimethylamine N-oxide on fluidity of liposomes and membranes of an elasmobranch. American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 276, R397-R406.

Beauchemin, K.A. (1991). Ingestion and mastication of feed by dairy cattle. In: C.J. Sniffen & T.H. Herdt (eds.) Dairy Nutrition Management. W.B. Saunders Co. Philadelphia, PA.

Benlamlih, S., A. Bourkaik, A. Belarbi & A. Abouhala (1985). Fluid balance in pregnant and lactating Dman ewes. Effect of litter size, protein intake and inhibition of prostaglandin synthesis. Comparative Biochemistry and Physiology A, 80, 591-597.

Benlamlih, S. & H. de Pomyers (1989). Changes in endogenous urea recycling and handling of renal urea in pregnant and lactating Sardi sheep kept at a constant feeding level. Reproduction Nutrition and Development, 29, 129-137.

Bloomfield, R.A., G.B. Garner & M.E. Muhrer (1960). Kinetics of urea metabolism in sheep. Journal of Animal Science, 19, 1248.

Bohra, H.C. & P.K. Ghosh (1983). Nitrogen metabolism in water restricted Marwari sheep of the Indian desert. Journal of Agricultural Science 101, 735-739.

Boivin, R. & J. Bost (1977). Ammonia et uree salivaire chez le mouton. Influence de surcharges alimentaires azotees (Saliva ammonia and urea in the sheep. Effects of alimentary nitrogen additives). Annales de Recherches Veterinaires, 8, 143-152.

Boldizarova, K., S. Faix & L. Leng (1999). The renal response of sheep fed a high protein diet to treatment with vasopressin analogue. Acta Veterinaria Brno, 68, 99-104.

Bradford, A.D., J. Terris, C.A. Ecelbarger, J.D. Klein, J.M. Sands, C.-L. Chou & M.A. Knepper. (2001). 97 and 117 kDa forms of the collecting duct urea transporter UT-A1 are due to different states of glycosylation. American Journal of Physiology Renal Physiology, 281, F133-F143.

Brook, A.H., H.M. Radford & B.D. Stacy (1968). Function of antidiuretic hormone in sheep. Journal of Physiology, 197 , 723-734.

Campbell, J.W. (1970). Comparative biochemistry of arginine and urea metabolism in invertebrates. In, B. Schmidt-Nielsen (ed.) Urea and the kidney. Excerpta Medical Foundation, Amsterdam. pp. 48-68.

Cheng, K.J. & R.J. Wallace (1979). The mechanism of passage of endogenous urea through the rumen wall and the role of ureolytic epithelial bacteria in the urea flux. British Journal of Nutrition, 42, 553-557.

Cirio, A. & R. Boivin (1990). Urea recycling from the renal pelvis in sheep, a study with 14C-urea. American Journal of Physiology, 258, F1196-F1202.

Cirio, A., R. Boivin & D. Grancher (1990). Diurese, debit plasmatique renal et filtration glomerulaire chez le mouton vigile et anesthesie, effets d'un regime carence en proteines (Urine flow, renal plasma flow and glomerular filtration in concious and anesthetized sheep – Effects of a low protein diet). Annales de Recherches Veterinaires, 21, 23-32.

Couriaud, C., P. Ripoche & G. Rousselet (1996). Cloning and functional characterization of a rat urea transporter: Expression in the brain. Biochimica Biophysica Acta Gene Structure and Expression, 1309, 197-199.

Cross, R.B., E. Lee & W.M. Thornton (1966). The effect of vasopressin on urea excretion by sheep. Journal of Physiology, 187, 34P-35P.

Denis, N., I. Tebot, J.M. Bonnet & A. Cirio (2004). Circadian rhythm of renal blood flow in caged sheep. Influence of eating and rumination. Journal of Animal and Feed Sciences, 13, suppl. 1, 311-314.

el Sayed, A.A., J. Haylor & A.M. el Nahas (1991). Mediators of the direct effects of amino acids on the rat kidney. Clinical Science, 81, 427-432.

Ergene, N. & E.C. Pickering (1978). The effect of urea infusion on glomerular filtration rate and renal plasma flow in sheep fed low and high protein diets. Quaterly Journal of Experimental Physiology and Cognate Medical Sciences, 63, 77-81.

Eriksson, L. & M. Valtonen (1982). Renal urea handling in goats fed high and low protein diets. Journal of Dairy Science, 65, 385-389.

Faichney, G.J. & G.A. White (1988). Partition of organic matter, fiber and protein digestion in ewes fed at a constant rate throughout gestation. Australian Journal of Agricultural Science, 39, 493-504.

Faix, S., L. Leng, M. Szanyiova & K. Boda (1988). Effect of dietary energy intake on tubular reabsorption of urea in sheep. Physiologia Bohemoslovaca, 37, 493-501.

Fenton, R.A., A. Howorth, G.J. Cooper, R. Meccariello, I.D. Morris & C.P. Smith (2000). Molecular characterization of a novel UT-A urea transporter isoform (UT-A5) in testis. American Journal of Physiology Cell Physiology, 279, C1425-C1431.

Fenton, R.A., C.A. Cottingham, G.S. Stewart, A. Howorth, J.A. Hewitt & C.P. Smith (2002a). Structure and characterization of the mouse UT-A gene (*Slc14a2*). American Journal of Physiology Renal Physiology, 282, F630-F638.

Fenton, R.A., G.S. Stewart, B. Carpenter, A. Howorth, E.A. Potter, G.J. Cooper & C.P. Smith (2002b). Characterization of the mouse urea transporters UT-A1 and UT-A2. American Journal of Physiology Renal Physiology, 283, F817-F825.

Fenton, R.A., C.L. Chou, G.S. Stewart, C.P. Smith & M.A. Knepper (2004). Urinary concentrating defect in mice with selective deletion of phloretin-sensitive urea transporters in the renal collecting duct. Proceedings of the National Academy of Sciences of the United States of America, 101, 7469-7474.

Gallucci, E., S. Micelli & C. Lippe (1975). Effect of cholesterol on the non-electrolyte permeability of planar lecithin membranes. Nature, 255, 722-723.

Goldstein, L. (1970). Urea metabolism and osmoregulation in euryhaline elasmobranches and amphibia. In Urea and the kidney, B. Schmidt-Nielsen, editor, Excerpta Medical Foundation, Amsterdam

Guyton, A.C. (1996). The Kidneys and Body Fluids. In: A.C. Guyton and J.E. Hall (eds.) Textbook of Medical Physiology. W.B. Saunders Company, Philadelphia, pp. 308-372.

Harmeyer J. & H. Martens (1980). Aspects of urea metabolism in ruminants with reference to the goat. Journal of Dairy Science, 63,1707-1728.

Harrop, C.J.F. (1974). Nitrogen metabolism in the ovine stomach. 2. Factors influencing ruminal ammonia levels in sheep maintained on different diets. Journal of Agricultural Science, 82, 409-417.

Harrop, C.J.F. & A.T. Phillipson (1974). Nitrogen metabolism in the ovine stomach. 1. The transfer of urea from the blood to the rumen. Journal of Agricultural Science, 82, 399-408.

Houpt, T.R. (1959). Utilization of blood urea in ruminants. American Journal of Physiology, 197, 115-120.

Houpt, T.R. & K.A. Houpt (1968). Transfer of urea nitrogen across the rumen wall. American Journal of Physiology, 214, 1296-1303.

Hu, M.C., L. Bankir, S. Michelet, G. Rousselet & M.M. Trinh-Trang-Tan (2000). Massive reduction of urea transporters in remnant kidney and brain of uremic rats. Kidney International, 58, 1202-1210.

Inoue, H., S.D. Jackson, T. Vikulina, J.D. Klein, K. Tomita & S.M. Bagnasco (2004). Identification and characterization of a Kidd antigen/UT-B urea transporter expressed in human colon. American Journal of Physiology Cell Physiology, 287, C30-C35.

Isozaki, T., A.G. Gillin, C.E. Swanson & J. M. Sands (1994). Protein restriction sequentially induces new urea transport processes in the rat initial IMCD. American Journal of Physiology, 266, F756-F761.

Jackson, A.A. (1998). Salvage of urea-nitrogen in the large bowel, functional significance in metabolic control and adaptation. Biochemical Society Transactions, 26, 231-236.

Karakashian, A., R.T. Timmer, J.D. Klein, R.B. Gunn, J.M. Sands & S.M. Bagnasco (1999). Cloning and characterization of two new mRNA isoforms of the rat renal urea transporter, UT-A3 and UT-A4. Journal of the American Society of Nephrology, 10, 230-237.

Kennedy, P.M. & L.P. Milligan (1980). The degradation and utilization of endogenous urea in the gastrointestinal tract of ruminants, a review. Canadian Journal of Animal Science, 60, 205-221.

Khan, M.S., T.O. Sasidharan & P.K. Ghosh (1979). Glomerular filtration rate and blood and urinary urea concentrations in Barmer goats of the Rajasthan desert. Experientia, 93, 247-248.

Kim, D.U., J.D. Klein, S. Racine, B.P. Murrell & J.M. Sands (2005) Urea may regulate urea transporter protein abundance during osmotic diuresis. American Journal of Physiology Renal Physiology 288, F188-F197.

Kim, H.S., J.J. Choung, D. Chamberlain & G.E. Lobley (1999). Effect of propionate on ovine urea kinetics. In: G.E. Lobley, A. White and J.C. MacRae (eds.) Protein Metabolism and Nutrition, European Association for Animal Production. Wageningen Press, P. 57.

Kim, Y.H., D.U. Kim, K.H. Han, J.Y. Jung, J.M. Sands, M.A. Knepper, K.M. Madsen & J. Kim (2002). Expression of urea transporters in the developing rat kidney. American Journal of Physiology Renal Physiology, 282, F530-F540.

King, A.J. & A.S. Levey (1993). Dietary protein and renal function. Journal of the American Society of Nephrology, 3, 1723-1737.

Klebl, F., M. Wolf & N. Sauer. (2003). A defect in the yeast plasma membrane urea transporter Dur3p is complemented by *CpNIP1*, a Nod26-like protein from zucchini (*Cucurbita pepo* L.), and by *Arabidopsis thaliana* d-TIP or gamma-TIP. Federation of European Biochemical Society Letters, 547, 69-74.

Klein, J.D., J.M. Sands, L. Qian, X. Wang & B. Yang (2004). Upregulation of urea transporter UT-A2 and water channels AQP2 and AQP3 in mice lacking urea transporter UT-B. Journal of the American Society of Nephrology, 15, 1161-1167.

Knepper, M.A. & F. Roch-Ramel (1987). Pathways of urea transport in the mammalian kidney. Kidney International, 31, 629-633.

Kwun, Y.S., S.W. Yeo, Y.H. Ahn, S.W. Lim, J.Y. Jung, W.Y. Kim, J.M. Sands & J. Kim (2003). Immunohistochemical localization of urea transporters A and B in the rat cochlea. Hearing Research, 183, 84-96.

Krehbiel, C.R., D.L. Harmon & J.E. Schmieder (1992). Effect of increasing ruminal butyrate on portal and hepatic nutrient flux in steers. Journal of Animal Science, 70, 904-914.

Lang, F., I. Ottl, D. Haussinger, P. Deetjen, M. Ahloulay & L. Bankir (1995). Renal hemodynamic response to intravenous and oral amino acids in animals. Seminars in Nephrology, 15, 415-418.

Lee, K.E. & R.A. Summerill (1982). Amino nitrogen metabolism following administration of individual amino acids or meat in conscious dogs. Quaterly Journal of Experimental Physiology, 67, 449-458.

Leng, L., M. Szanyiova & K. Boda (1985). The renal response of sheep to a low dietary nitrogen intake. Physiologia Bohemoslovaca, 34, 147-159.

Leng, L., M. Szanyiova, S. Faix & A. Cirio (1994). Renal response of sheep fed a low protein diet to intraportal infusion of arginine and glycine. Comparative Biochemistry and Physiology A, 108, 343-347.

Leung, D.W., D.D.F. Loo, B.A. Hirayama, T. Zeuthen & E.M. Wright (2000). Urea transport by cotransporters. Journal of Physiology (London), 528, 251-257.

Liu, L.H., U. Ludewig, W.B. Frommer, and N. Von Wiren (2003). AtDUR3 encodes a new type of high-affinity urea/H^+ symporter in Arabidopsis. Plant Cell, 15, 790-800.

Lobley, G.E. & H. Lapierre (2001). Nitrogen recycling in the ruminant: A review. Journal of Dairy Science, 84, E223-E236.

Lucien, N., F. Sidoux-Walter, B. Olives, J. Moulds, P.Y. Le Pennec, J.P. Cartron & P. Bailly (1998). Characterization of the gene encoding the human Kidd blood group urea transporter protein - Evidence for splice site mutations in Jk_{null} individuals. Journal of Biological Chemistry, 273, 12973-12980.

Lucien, N., F. Sidoux-Walter, N. Roudier, P. Ripoche, M. Huet, M.M. Trinh-Trang-Tan, J.P. Cartron, and P. Bailly (2002). Antigenic and functional properties of the human red blood cell urea transporter hUT-B1. Journal of Biological Chemistry, 277, 34101-34108.

Macey, R.I. 1984. Transport of water and urea in red blood cells. American Journal of Physiology Celll Physiology, 246, C195-C203.

Maltz, E. & N. Silanikove (1996). Kidney function and nitrogen balance of high yielding dairy cows at the onset of lactation. Journal of Dairy Science, 79, 1621-1626.

Maltz, E., N. Silanikove & A. Shkolnik (1981). Renal performance in relation to water and nitrogen metabolism in Bedouin goats during lactacion. Comparative Biochemistry and Physiology A, 70, 145-147.

Marini, J.C. (1998). Effect of intravenous infusion of urea on nitrogen metabolism of steers. Master Thesis. Iowa State University.

Marini, J.C., J.D. Klein, J.M. Sands & M.E. Van Amburgh (2004). Effect of nitrogen intake on nitrogen recycling and urea transporter abundance in lambs. Journal of Animal Sciences, 82, 1157-1164.

Marini, J.C. & M.E Van Amburgh (2003). Nitrogen metabolism and recycling in Holstein heifers. Journal of Animal Sciences, 81, 545-552.

Minocha, R., K. Studley, and M.H. Saier, Jr (2003). The urea transporter (UT) family: bioinformatic analyses leading to structural, functional, and evolutionary predictions. Receptors and Channels, 9, 345-352.

Mooney, P. & D.J. O'Donovan (1970). The permeability of the rumen to simple nitrogenous compounds. Biochemical Journal, 119, 18p-19p.

Mousa, H.M., K.E. Ali & I.D. Hume (1983). Effects of water deprivation on urea metabolism in camels, desert sheep and desert goats fed dry desert grass. Comparative Biochemistry and Physiology A, 74, 715-720.

Nakayama, Y., M. Naruse, A. Karakashian, T. Peng, J.M. Sands, and S.M. Bagnasco (2001). Cloning of the rat Slc14a2 gene and genomic organization of the UT-A urea transporter. Biochimica Biophysica Acta, 1518, 19-26.

Ndibualonji, B.B., M.N. Rodriguez, D. Dehareng, A. Cirio & J.M. Godeau (1998). Effects of late pregnancy and early lactation on renal urea handling in Corriedale ewes. Journal of Agricultural Science, 130, 213-216.

Nielsen S., J. Frokiaer, D. Marples, E.D. Kwon, P Agre and M. Knepper. (2002) Aquaporins in the Kidney: From Molecules to Medicine. Physiological Reviews, 82, 205-244.

Nielsen, S., J. Terris, C.P. Smith, M.A. Hediger, C.A. Ecelbarger, and M.A. Knepper. 1996. Cellular and subcellular localization of the vasopressin-regulated urea transporter in rat kidney. Proceedings of the National Academy of Sciences of the United States of America, 93, 5495-5500.

Nolan, J.V. & R.A. Leng (1970). Metabolism of urea in late pregnancy and possible contribution of amino acid carbon to glucose synthesis in sheep. British Journal of Nutrition, 24, 905-915.

Nolan, J.V., B.W. Norton & R.A. Leng (1973). Nitrogen cycling in sheep. Proceedings of the Nutrition Society, 32, 93-98.

Norton, B.W., A.N. Janes & D.G. Armstrong (1982). The effects of intraruminal infusions of sodium bicarbonate, ammonium chloride and sodium butyrate on urea metabolism in sheep. British Journal of Nutrition, 48, 265-274.

O'Connor, W.J. & R.A. Summerill (1976). The effect of a meal of meat on glomerular filtration rate in dogs at normal urine flows. Journal of Physiology, 256, 81-91.

Olives, B., S. Martial, M.G. Mattei, G. Matassi, G. Rousselet, P. Ripoche, J.P. Cartron, and P. Bailly (1996). Molecular characterization of a new urea transporter in the human kidney. Federation of European Biochemical Society Letters, 386, 156-160.

Olives, B., M.G. Mattei, M. Huet, P. Neau, S. Martial, J.P. Cartron, and P. Bailly (1995). Kidd blood group and urea transport function of human erythrocytes are carried by the same protein. Journal of Biological Chemistry, 270, 15607-15610.

Olives, B., P. Neau, P. Bailly, M.A. Hediger, G. Rousselet, J.P. Cartron, and P. Ripoche (1994). Cloning and functional expression of a urea transporter from human bone marrow cells. Journal of Biological Chemistry, 269, 31649-31652.

Pallone, T.L (1994). Characterization of the urea transporter in outer medullary descending vasa recta. American Journal of Physiology Regulatory, Integrative, and Comparative Physiology, 267, R260-R267.

Pallone, T.L., S. Nielsen, E.P. Silldorff, and S. Yang (1995). Diffusive transport of solute in the rat medullary microcirculation. American Journal of Physiology Renal Physiology, 269, F55-F63.

Pallone, T.L., J. Work, R.L. Myers, and R.L. Jamison (1994). Transport of sodium and urea in outer medullary descending vasa recta. Journal of Clinical Investigation, 93, 212-222.

Panayotova-Heiermann, M. and E.A. Wright (2001). Mapping the urea channel through the rabbit Na^+-glucose cotransporter SGLT1. Journal of Physiology (London), 535, 419-425.

Pearson, R.M. & J.A.B. Smith (1943). The utilization of urea in the bovine rumen. 2. The conversion of urea to ammonia. Biochemical Journal, 37, 148-153.

Phillips, G.D., E.C. Pickering & K. Wilkinson (1975). Renal responses of the cow to alteration of the dietary intake of nitrogen and sodium chloride. Journal of Physiology, 245, 95P-96P.

Ripoche, P. & G. Rousselet (1996). Les transporteurs de l'urée (The urea transporters). Nephrologie, 17, 383-388.

Ritzhaupt, A., G. Breves, B. Shroeder, C.G. Winckler & S.P. Shirazi-Beechey (1997). Urea transport in gastrointestinal tract of ruminants, effect of dietary nitrogen. Biochemical Society Transactions, 25, 490S.

Ritzhaupt, A., I.S. Wood, A.A. Jackson, B.J. Moran & S.P. Shirazi-Beechey (1998). Isolation of a RT-PCR fragment from human colon and sheep rumen RNA with nucleotide sequence similarity to human and rat urea transporter isoforms. Biochemical Society Transactions, 26, S122 .

Rundgren, M., S. Eriksson & B. Appelgren (1979). Urea-induced inhibition of antidiuretic hormone (ADH) secretion. Acta Physiologica Scandinavica, 106, 491-492.

Sands, J.M. (1999). Regulation of renal urea transporters. Journal of the American Society of Nephrology, 10, 635-646.

Sands, J. M. (2003a). Mammalian urea transporters. Annual Review of Physiology, 65, 543-566.

Sands, J. M. (2003b). Molecular mechanisms of urea transport. Journal of Membrane Biology, 191, 149-163.

Sands, J. M. (2004). Renal urea transporters. Current Opinion in Nephrology and Hypertension, 13, 525-532

Sands, J.M., J.J. Gargus, O. Frohlich, R.B. Gunn & J.P. Kokko (1992). Urinary concentration ability in patients with JK (a-b-) blood type who lack carrier-mediated urea transport. Journal of the American Society of Nephrology, 2, 1689-1696.

Sands, J.M., H. Nonoguchi, and M.A. Knepper (1987). Vasopressin effects on urea and H_20 transport in inner medullary collecting duct subsegments. American Journal of Physiology Renal Physiology, 253, F823-F832.

Sands, J.M., R.T. Timmer, and R.B. Gunn (1997). Urea transporters in kidney and erythrocytes. American Journal of Physiology Renal Physiology, 273, F321-F339.

Schmidt-Nielsen, B. & H. Osaki (1958). Renal response to changes in nitrogen metabolism in sheep. American Journal of Physiology, 193, 657-661.

Schmidt-Nielsen, B., H. Osaki, H.V. Murdaugh & R. Odell (1958). Renal regulation of urea excretion in sheep. American Journal of Physiology, 194, 221-228.

Shayakul, C. & M. A. Hediger (2004). The SLC14 gene family of urea transporters. Pfluegers Archives, 447, 603-609.

Sidoux-Walter, F., N. Lucien, R. Nissinen, P. Sistonen, S. Henry, J. Moulds, J.P. Cartron, and P. Bailly (2000). Molecular heterogeneity of the Jk_{null} phenotype: expression analysis of the Jk(S291P) mutation found in Finns. Blood, 96, 1566-1573.

Silanikove, N. (1986). Interrelationships between feed quality, digestibility, feed consumption, and energy requirements in desert (Bedouin) and temperate (Saanen) goats. Journal of Dairy Science, 69, 2157-2162.

Somers, M. (1961a). Factors influencing the secretion of nitrogen in the sheep saliva. 1. The distribution of nitrogen in the mixed and parotid saliva of sheep. Australian Journal of Experimental Biology and Medical Science, 39, 111-122.

Somers, M. (1961b). Factors influencing the secretion of nitrogen in the sheep saliva. 2. The influence of nitrogen intake upon blood urea nitrogen and upon the total nitrogen and urea nitrogen in the parotid saliva of sheep. Australian Journal of Experimental Biology and Medical Science, 39, 123-132.

Somers, M. (1961c). Factors influencing the secretion of nitrogen in the sheep saliva. 3. Factors affecting the nitrogen fractions in the parotid saliva of sheep with special reference to the influence of ammonia production in the rumen and fluctuation in levels of blood urea. Australian Journal of Experimental Biology and Medical Science, 39, 133-144.

Spector, D.A., Q. Yang, J. Liu & J.B. Wade (2004). Expression, localization, and regulation of urea transporter B in rat urothelia. American Journal of Physiology Renal Physiology, 287, F102-F108.

Stewart, G.S., R.A. Fenton, F.Thévenod & C.P. Smith (2004a). Urea movement across mouse colonic plasma membranes is mediated by UT-A urea transporters. Gastroenterology, 126, 765-773.

Stewart, G.S., R.A. Fenton, W.Wang, T.H. Kwon, S.J. White, V.M. Collins, G. Cooper, S. Nielsen & C.P. Smith (2004b). The basolateral expression of mUT-A3 in the mouse kidney. American Journal of Physiology Renal Physiology, 286, F979-F987.

Stewart, G.S., C. Graham, S. Cattell, T.P.L., N.L. Simmons &C.P. Smith (2004). Identification and localization of a UT-B facilitative urea transporter in bovine rumen. Submitted (MAY-2004) to the EMBL/GenBank/DDBJ databases.

Sutoh, M., Y. Obara & S. Miyamoto (1994). Effects on nitrogen kinetics in sheep of the supplementation of sodium propionate and sodium acetate. Animal Science and Technology, 65, 593-601.

Tebot, I., S. Faix, M. Szanyiova, A. Cirio & L. Leng (1998). Micropuncture study on urea movements in the kidney cortical tubules of low protein fed sheep. Veterinary Research, 29, 99-105.

Terris, J.M., M.A. Knepper & J.B. Wade (2001). UT-A3: localization and characterization of an additional urea transporter isoform in the IMCD. American Journal of Physiology Renal Physiology, 280, F325-F332.

Thorlacius, S.O., A. Dobson & A.F. Sellers (1971). Effect of carbon dioxide on urea diffusion through bovine ruminal epithelium. American Journal of Physiology, 220, 162-170.

Thornton, R.F. (1970). Urea excretion in ruminants. I. Studies in sheep and cattle offered the same diet. Australian Journal of Agricultural Research, 21, 323-336.

Timmer, R.T., J.D. Klein, S.M. Bagnasco, J.J. Doran, J.W. Verlander, R.B. Gunn, and J.M. Sands (2001). Localization of the urea transporter UT-B protein in human and rat erythrocytes and tissues. American Journal of Physiology Cell Physiology, 281, C1318-C1325.

Trinh-Trang-Tan, M.M., N. Bouby, W. Kriz & L. Bankir (1987). Functional adaptation of thick ascending limb and internephron heterogeneity to urine concentration. Kidney International, 31,5 49-555.

Tsukaguchi, H., C. Shayakul, U. V. Berger, T. Tokui, D. Brown & M. A. Hediger (1997). Cloning and characterization of the urea transporter UT3. Localization in rat kidney and testis. Journal of Clinical Investigation, 99, 1506-1515.

Utley, P.R., N.W. Bradley& J.A. Boling (1970a). Effect of water restriction on nitrogen metabolism in bovine fed two levels of nitrogen. Journal of Nutrition, 100, 551-556.

Utley, P.R., N.W. Bradley & J.A. Boling (1970b). Effect of restricted water intake on feed intake nutrient digestibility and nitrogen metabolism in steers. Journal of Animal Science, 31, 130-135.

Varady, J., K.T. Tashenov, K. Boda, J. Fejes & K. Kosta (1979). Endogenous urea secretion into the sheep gastrointestinal tract. Physiologia Bohemoslovaca, 28, 551-559.

Vercoe, J.E. (1969). The transfer of nitrogen from the blood to the rumen of cattle. Australian Journal of Agricultural Research, 20, 191-197.

Wade, J.B., A.J. Lee, J. Liu, C.A. Ecelbarger, C. Mitchell, A.D. Bradford, J. Terris, G.H. Kim, and M.A. Knepper (2000). UT-A2: a 55 kDa urea transporter protein in thin descending limb of Henle's loop whose abundance is regulated by vasopressin. American Journal of Physiology Renal Physiology, 278, F52-F62.

Wallace, R.J., K.J. Cheng, D. Dinsdale & E.R. Orskov (1979). An independent microbilal flora of the epithelium and its role in the ecomicrobiology of the rumen. Nature, 279, 424-426.

Wells, R.M.G., R.H. McIntyre, A.K. Morgan & P.S. Davie (1986). Physiological stress responses in big gamefish after capture, observations on plasma chemistry and blood factors. Comparative Biochemistry and Physiology, 84A, 565-571.

Whitelaw, F.G., J.S. Milne & S.A. Wright (1991). Urease (EC 3.5.1.5) inhibition in the sheep rumen and its effect on urea and nitrogen metabolism. British Journal of Nutrition, 66, 209-225.

Wilcox, C.S. & W.J. Welch (1998). Macula densa nitric oxide synthase, expression, regulation, and function. Kidney International, 67, S53-S57.

Xu, Y., B. Olives, P. Bailly, E. Fischer, P. Ripoche, P. Ronco, J.P. Cartron, and E. Rondeau (1997). Endothelial cells of the kidney vasa recta express the urea transporter HUT11. Kidney International, 51, 138-146.

Yang, B., L. Bankir, A. Gillespie, C.J. Epstein & A.S. Verkman (2002). Urea-selective concentrating defect in transgenic mice lacking urea transporter UT-B. Journal of Biological Chemistry, 277, 10633-10637.

Yesberg, N.E., M. Henderson & O.E. Budtzololsen (1970). Excretion of vasopressin by normal and dehydrated sheep. Australian Journal of Experimental Biology and Medical Science, 48, 115-127.

You, G., C.P. Smith, Y. Kanai, W.S. Lee, M. Stelzner & M.A. Hediger (1993). Cloning and characterization of the vasopressin-regulated urea transporters. Nature, 365, 844-847.

Ruminal SCFA absorption: channelling acids without harm

G. Gäbel and J.R. Aschenbach
Institute of Veterinary Physiology, Leipzig University, An den Tierkliniken 7, D-04103 Leipzig, Germany

Abstract

Several studies indicate that the bulk absorption of short chain fatty acids (SCFA) occurs in the protonated forms (HSCFA). However, there are strong hints that SCFA are also taken up in their dissociated forms ($SCFA^-$) by anion exchange systems in the apical membrane. On a molecular basis, various transport proteins (anion exchange / AE; putative anion transport / PAT; down-regulated in adenoma / DRA) are characterized which may accept SCFA in their dissociated form. After luminal uptake, SCFA can either be catabolized or extruded on the basolateral (blood-oriented side) of the cell. Intracellular catabolism leads to ketonic acids (acetoacetate, D-3-hydroxybutyrate) and lactate. These monocarboxylates are rather hydrophilic and need specific transport proteins. Recent functional, structural and immunocytochemical studies revealed a monocarboxylate transporter isoform 1 (MCT1) in the stratum basale, which most likely mediates the effective elimination of the ketonic acids and lactic acid into the direction of blood.

Both uptake of HSCFA and exchange of intracellular HCO_3^- for extracellular $SCFA^-$ acidify the cytosol. The rumen epithelium has developed sophisticated strategies to eliminate the surplus of protons. The main proton exporting mechanisms seem to be a sodium / proton exchanger isoform 1 (NHE1) and the MCT1 on the basolateral side of the epithelium. Proton extrusion is supported by import of HCO_3^- via a sodium bicarbonate cotransporter isoform 1 (NBC1).

Keywords: Intracellular pH, SCFA transport, catabolism, anion exchange, monocarboxylate transport

Introduction

The importance of absorptive processes in the rumen for electrolyte and energy balance of the animal is well accepted and has been reviewed several times in ISRP (Gäbel and Martens, 1991; Rechkemmer *et al*., 1995). For example, the ruminal epithelium is crucial for the balance of sodium (Martens and Gäbel, 1988; Gäbel and Martens, 1991), magnesium (Leonhard-Marek, 1999; Martens and Schweigel, 2000), bicarbonate (Gäbel *et al*., 1991a), and short chain fatty acids (Rechkemmer *et al*., 1995; Gäbel *et al*., 2002). Out of these, the transruminal permeation of short chain fatty acids has to be regarded the main absorptive process from a quantitative point of view. The large production and absorption of SCFA in the rumen implies great acid-base shifts between different compartments (ruminal content, ruminal epithelium, systemic circulation; Gäbel *et al*., 2002). This review is intended to summarize the current knowledge on the quantitative and qualitative aspects of SCFA transfer across the ruminal epithelium with due consideration of their impact on acid-base equilibrium. Special attention will be given to the intracellular conversions of acids (i.e. metabolism), the interplay of acids and bicarbonate, and the regulation of intracellular pH (pH_i) in ruminal epithelial cells.

Quantitative aspects of intraruminal acid production and their final fate

The intraruminal net production rate of total SCFA (i.e. microbial production minus microbial utilisation) was estimated to be approximately 5 mol/kg DM intake on one day (Bergman, 1990). Depending on the type of diet and the intake of organic matter, up to 85% of the acids produced can be taken up by the ruminal epithelium. (Sutherland, 1963; Tamminga and van Vuuren 1988; Peters *et al*., 1990, 1992; Dijkstra *et al*., 1993; Lopez *et al*., 2003). On the example of propionate, it has been shown that the majority (~95%) of SCFA entering the omasum never reach the small intestine (Peters *et al*., 1990). Therefore, the absorption of ruminally produced SCFA is a process that is almost complete at the pylorus of the abomasum. Out of the 5 mol SCFA that are absorbed per kg DM intake, only 4.2 mol do net appear in the portal circulation (Kristensen *et al*., 1996). This implies a metabolic loss of about 15% of SCFA in the portal tissues, most of it being butyrate (Kristensen *et al*., 2000a, b). Portal acetate and butyrate are delivered mainly to the liver and mammary gland for lipogenesis, whereas propionate is mainly used for hepatic gluconeogenesis.

Intraruminal proton release and buffering

If ~5 mol of SCFA are net produced in the rumen per kg of dry matter intake, > 4.6 mol H^+ are released in parallel at a ruminal pH > 6. A small fraction of these protons would be sufficient to induce a fatal drop in the ruminal pH. The pH drop during periods of intensive fermentation, however, is usually very mild. This has long been attributed solely to proton buffering by salivary secretion of HCO_3^- and HPO_4^{2-}. However, according to calculations by Ash and Dobson (1963) and Ørskov (1995), inflow of saliva would be sufficient to neutralize only 15-20% of the SCFA produced, leading to an intraruminal pH below 4.5. Since the physiological pH of the ruminal content is about 6.5, there have to exist neutralization pathways other than saliva. These neutralizing mechanisms are mainly absorption of undissociated SCFA and secretion of HCO_3^- by the ruminal wall. Regarding the latter, it can be estimated that roughly 0.5 mol HCO_3^- is ruminally secreted for 1 mol SCFA absorbed (Gäbel *et al*., 1991a).

Mechanisms of acid elimination from the ruminal content

Over the last years, the model for the elimination of SCFA from the rumen has become more and more sophisticated. The current knowledge is outlined in Figure 1. SCFA exist in a protonated form (HSCFA) and an ionized form ($SCFA^-$). HSCFA are highly lipid-soluble and, consequently, may permeate biological membranes directly (Leo *et al*., 1971; Walter and Gutknecht, 1986). However, the effects of pH and lipophilicity on SCFA absorption are only small (review in Gäbel *et al*., 2002), supporting the view that SCFA cross the ruminal epithelium not only by passive diffusion in their undissociated forms.

The dissociated forms of SCFA are not lipophilic and, therefore, need specific transport mechanisms. The apical presence of such transport systems for $SCFA^-$ was first suggested by the observations of Ash and Dobson (1963) that (1) HCO_3^- is efficiently secreted into the lumen and (2) HCO_3^- secretion is dependent on the presence and/or absorption of SCFA. SCFA seem to be essential to drive HCO_3^- secretion in the rumen and can hardly or not be replaced by chloride or

lactate (Gäbel *et al*., 1991a). The findings enforced the hypothesis of transport proteins working as anion exchangers, i.e., exchanging absorbed $SCFA^-$ for secreted HCO_3^- (Gäbel *et al*., 1991a). Although this hypothesis was expressed some years ago, the properties of the anion exchange system(s) are still under debate.

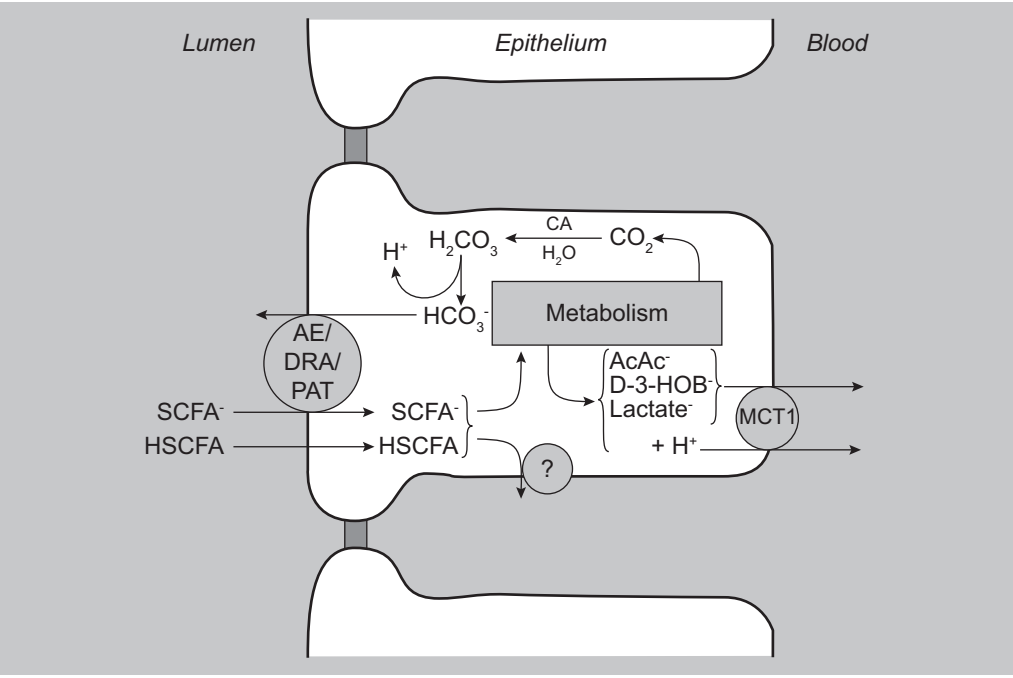

Figure 1. Transport of SCFA across ruminal epithelium.

Apical uptake of SCFA surely occurs via diffusion of undissociated SCFA (HSCFA). Additional absorption of dissociated SCFA ($SCFA^-$) by anion exchange proteins (AE, DRA, or PAT) is suggested by functional studies in vivo and in vitro (Ash and Dobson, 1963; Gäbel et al.*, 1991b; Kramer* et al.*, 1996; Sehested* et al.*, 1999a,b) and by molecular biological studies. After uptake, SCFA are partially metabolized through oxidative or anaerobic pathways. Carbon dioxide from the oxidative breakdown can be further transformed by the activity of carboanhydrase (CA) to H_2CO_3 which in turn dissociates into HCO_3^- and H^+. HCO_3^- may drive the apical anion exchangers. Fate of H^+ is outlined in Figure 2. Ketone bodies ($AcAc^-$, acetoacetate; $D\text{-}3\text{-}HOB^-$, D-3-hydroxybutyrate) and lactate derived from the anaerobic breakdown can be extruded on the basolateral side by the proton-linked monocarboxylate transporter MCT1. Basolateral extrusion of SCFA partly takes places by simple diffusion of HSCFA. If and which anion exchange proteins or other transport proteins are involved has to be elucidated.*

Besides MCT1, the localisation of transport proteins is so far only derived from functional studies and from comparisons with other gastrointestinal epithelia. MCT1 has been localized by immunocytochemical methods (Figure 6). The multilayered structure of the rumen epithelium is reduced to one layer in the model presented. Consequently, the "basolateral membrane" represents the blood oriented side of the cells in the stratum basale. The "apical membrane" is located on the lumen oriented side of the cells in the upper layers of the epithelium

Comparison with other gastrointestinal segments might be helpful to get an idea as to the nature of anion exchange in the rumen. Effective HCO_3^- secretion can be found in other gastrointestinal (GI) epithelia, too. In the duodenum, HCO_3^- secretion protects the mucosa against HCl inflow with the gastric juice (Flemstrom, 1994; Holm *et al*., 1998; Wang *et al*., 2002). However, HCO_3^- secretion in the duodenum is probably not a good example to clarify the mechanisms in the rumen. In the duodenum, SCFA are hardly present and, in addition, duodenal HCO_3^- secretion seems to be mainly coupled to Cl^- (Wang *et al*., 2002; Jacob *et al*., 2002). The colon and caecum, on the other hand, are also GI segments with microbial fermentation and show several functional similarities to the rumen. Thus, a protein functioning as $SCFA^-/HCO_3^-$ exchanger would be likely to occur in the large intestine as well. There is already functional evidence for this type of anion exchange in the colon of rat and men (Reynolds *et al*., 1993; Harig *et al*., 1996; Sellin, 1999; Rajendran and Binder 2000; Rajendran *et al*., 2000; Vidyasagar *et al*., 2004).

Several transporter families could contain the molecular correlate of functional $SCFA^-/HCO_3^-$ exchange. One of these is the family of anion exchangers (AE; review in Alper *et al*., 2002). In addition to AE, DRA (down-regulated in adenoma) proteins and/or PAT (putative anion transporter) proteins have recently been demonstrated as structural equivalents for the Cl^-/HCO_3^- exchange in the duodenum (Jacob *et al*., 2002; Wang *et al*., 2002). AE and DRA proteins have also been found in the membrane of rat colonic epithelial cells (Rajendran and Binder, 2000; Ikuma *et al*., 2003). However, an ability to function as $SCFA^-/HCO_3^-$ exchangers has not been demonstrated for either of these transporters so far.

Regarding the rumen, mRNA transcripts for AE2, DRA and PAT1 have recently been identified in the ovine ruminal epithelium as well as in cultured ruminal cells (Bilk *et al*., 2005). Similarly to the situation in the colon, however, structure-function relationships are not yet clarified. It is still not known which anions are preferably transported by the ruminal AE2, DRA and PAT1 proteins and – especially - whether any of the transporters identified on the molecular level may really serve as $SCFA^-/HCO_3^-$ exchanger. It is likely that some of these transporters function as Cl^-/HCO_3^- exchangers and are involved in electrically silent absorption of NaCl from the rumen (Martens and Gäbel, 1988). Experimental data suggests that Cl^-/HCO_3^- exchange, which carries the chloride part of NaCl absorption, is mediated by transporters different from those that mediate $SCFA^-/HCO_3^-$ exchange (Kramer *et al*., 1996). To complete the functional model, therefore, the transport properties of the identified anion exchangers and their localization within the stratified ruminal epithelium need to be elucidated.

Intraepithelial metabolism of SCFA

Following absorption, SCFA are partly metabolized within the epithelium (Bergman, 1990). Intraepithelial metabolism of SCFA was confirmed in a number of recent studies (Sehested *et al*., 1999a, b; Kristensen *et al*., 2000a, b). The findings on the extent of intraepithelial breakdown of acetate and propionate are controversial. Bergman and Wolff (1971) suggested that the ruminal epithelium metabolized 30, 50, and 90% of ruminally absorbed acetate, propionate, and butyrate, respectively. However, that would suggest that epithelial catabolism of SCFA produces more ATP than needed (Kristensen and Harmon, 2004). Complex *in vivo* studies combining several techniques support the suggestion that only n-butyrate is broken down to a great extent

(Kristensen and Harmon, 2004). The metabolism of n-butyrate may reach 95% (Bergman, 1990; Sehested *et al*., 1999a,b; Kristensen *et al*., 2000a,b; Noziere *et al*., 2000; Kristensen and Harmon, 2004; Table 1). Preferential catabolism of n-butyrate can be partially explained by differences in the epithelial ability to activate the three SCFA. Activation by formation of CoA esters is the initial step of SCFA breakdown in the ruminal epithelial cell. Different short chain acyl-CoA synthetases are involved in this activation (Weekes, 1971, 1972; Scaife and Tichivangana, 1980; Harmon *et al*., 1991). Each acyl-CoA synthetase shows specific substrate binding and kinetic properties (Scaife and Tichivangana, 1980; Harmon *et al*., 1991). According to the different enzyme kinetics, the three SCFA are activated in the order acetate < propionate < n-butyrate (Scaife and Tichivangana, 1980). Acetate and propionate activation in the ruminal epithelium is even further depressed in the presence of n-butyrate, whereas activation of n-butyrate seems to be rather unaffected by the co-presence of acetate or propionate (Ash and Baird, 1973; Scaife and Tichivangana, 1980; Harmon *et al*., 1991). Consequently, both the specific kinetics described above and the competition between the SCFA lead to preferential activation (and metabolism) of n-butyrate.

As shown in Figure 1, SCFA breakdown can be both aerobically to CO_2 and anaerobically to acetoacetate, D-3-hydroxybutyrate and lactate. Aerobic catabolism seems to play a minor role. According to studies in fistulated steers (Kristensen and Harmon, 2004), n-butyrate is seemingly not oxidized to CO_2 but anaerobically meta- and catabolized into acetate, ketone bodies and other products. The small fraction of aerobically catabolized n-butyrate may additionally be diminished by the co-presence of glucose (Giesecke *et al*., 1979, 1985; Baldwin and Jesse, 1992).

Disadvantages vs. advantages of intraepithelial SCFA breakdown

The intraepithelial breakdown of SCFA has no obvious advantages as far as acid-base balance is concerned. It could be advantageous if the acids would be degraded aerobically to CO_2. The latter could leave the cell via diffusion and additionally, after carbonic anhydrase reaction, as HCO_3^- and H^+ (Figure 1). However, as stated above, the aerobic pathway of SCFA catabolism is rather poorly used by ruminal epithelial cells. Instead, acidic substrates with a low lipophilicity are produced like acetoacetate, D-3-hydroxybutyrate and lactate (Table 2).

Table 1. Portal recovery of SCFA absorbed from the washed rumen of steers filled with buffer solutions containing bicarbonate (Source: data from Kristensen and Harmon, 2004).
[2-^{13}C]Acetate corrected absorption was calculated as the net portal flux + portal drained uptake of arterial acetate.

	Fractional portal recovery
Acetate	0.71
[2-^{13}C]Acetate corrected absorption	1.06
Propionate	0.91
Butyrate	0.27
Isovalerate	0.54
Valerate	0.30

Table 2. pK values and octanol/water partition coefficients ($logK_{Oct/H2O}$; ion-corrected) of ketonic acids and lactic acid compared to n-butyric acid (Source: pK-values are from Rapoport and Radebrecht, 1977; $K_{Oct/H2O}$- values are from Leo et al.*, 1971).*

	pK	$logK_{Oct/H2O}$
n-Butyric acid	4.8	0.79
Lactic acid	3.8	-0.62
D-3-hydroxybutyric acid	4.8	-0.23
Acetoacetic acid	3.8	--

These hydrophilic substrates cannot leave the cell by simple diffusion. Additionally, some of these hydrophilic substrates have a very low pK value (Table 2) and thereby decrease the intracellular buffer capacity. SCFA breakdown has thus the potential to disturb pH_i homeostasis, which will be discussed in context with pH_i regulation later in this review. From other perspectives, intracellular SCFA catabolism undoubtedly offers advantages for the animal: epithelial functions are energized, apical uptake of SCFA is improved, energy substrates are delivered to the animals, and deleterious butyrate accumulation may be prevented.

As far as energy for epithelial functions is concerned, one needs to consider that the forestomach is a main absorptive organ for major nutrients, e.g. SCFA, Na, and Mg. Consequently, the forestomach wall has the greatest energy requirement of viscera, the latter constituting about 25% of total body energy metabolism (Huntington, 1990; Britton and Krehbiel, 1993; Rémond *et al*., 1995). Glucose is principally available from arterial sources to cover the high energy demand of ruminal epithelial cells. However, net glucose disappearance from the blood into the ruminal epithelium is rather low (e.g. 6-12 mmol/d in adult sheep on a hay-based diet; Rémond *et al*., 1993, 1995; Han *et al*., 2002). This can be attributed to a preference for SCFA metabolism even in the presence of glucose (Rémond *et al*., 1995; Baldwin and Jesse, 1992; Britton and Krehbiel, 1993). Teleologically, the metabolic consumption by the ruminal tissue of SCFA instead of glucose makes sense because glucose is largely derived from gluconeogenesis in ruminants and, therefore, a rather 'expensive' fuel.

Improving the absorption rate of SCFA is probably the second advantage of intraepithelial SCFA catabolism. Stimulating effects of metabolism on absorption can be deduced from comparisons between the absorption rates of SCFA isoforms. The iso-butyrate and iso-valerate have similar lipophilicities like their n-forms (Leo *et al*., 1971) but they are catabolized to a lesser extent (Kristensen *et al*., 1996, 2000a, b; Kristensen and Harmon, 2004). Regarding absorption rates, net transport rates of iso-butyrate and -valerate are smaller than those of the respective n-forms (Weigand *et al*., 1975; Charney *et al*., 1998; Kristensen *et al*., 2000b; Gäbel *et al*., 2001; Kristensen and Harmon, 2004). This points to dependence of transport rates on the extent of metabolism. Different affinities of the iso- and n-forms to the putative $SCFA^-/HCO_3^-$ exchanger would also provide an explanation for the differences in absorption rates. However, the latter option is less likely in the light of studies with blockage of intracellular metabolism. In isolated ruminal epithelia, ATP depletion induced by tissue anoxia or addition of 2, 4-dinitrophenol diminished

the disappearance of SCFA from the luminal side and the net transport of n-butyrate to the blood side (Stevens and Stettler, 1966; Gäbel *et al*., 2001).

The two explanations for the interaction between metabolism and absorption are outlined in Figure 1. On the one hand, intraepithelial metabolism increases the concentration gradient between lumen and cytosol. This favours both non-ionic diffusion of HSCFA and anion exchange of $SCFA^-$ into the cell. On the other hand, the CO_2 produced during intracellular oxidative breakdown of SCFA can, via H_2CO_3, be converted to HCO_3^- with the aid of carbonic anhydrase located in the ruminal wall (Carter, 1971; Asari *et al*., 1989; Amasaki *et al*., 1991). Produced HCO_3^- would then drive the anion exchange mechanism already mentioned above. Supporting the latter assumption, *in vitro* studies by Sehested *et al.* (1999a) evidenced a clear preference for a luminally directed excretion of CO_2 (or HCO_3^-) derived from the intraepithelial SCFA metabolism. *In vivo*, however, only minor amounts of SCFA seem to be aerobically converted to CO_2 (Kristensen and Harmon, 2004). Consequently, only minor quantities of CO_2 (or HCO_3^-) would be available to drive apical SCFA uptake. The promoting influence of metabolism on SCFA absorption would then be mainly due to improving the concentration gradient across the apical membrane.

Besides delivering energy and driving apical uptake of SCFA, the early catabolism of SCFA to ketone bodies seems to offer a third advantage. Ketone bodies released by the ruminal epithelium are able to bypass the liver (Giesecke *et al*., 1985). These bypassed ketone bodies can serve as energy supply for extra-hepatic tissues (heart, striated muscle, adipose tissue, kidney, and mucosal cells; Robinson and Williamson, 1980; Voet and Voet, 1992; Britton and Krehbiel, 1993; Halestrap and Price, 1999). Moreover, D-3-hydroxybutyrate, together with acetate, is an important precursor of milk fatty acids in the lactating mammary gland (Robinson and Williamson, 1980). Therefore, peripherally circulating ketone bodies have to be regarded as valuable metabolites for energy metabolism and milk synthesis. In contrast to their precursors, the fatty acids, they have the additional advantage of being well water-soluble (Voet and Voet, 1992).

That n-butyrate (but not acetate or propionate) is preferentially catabolized has to be regarded as a fourth advantage of intraepithelial SCFA metabolism. n-Butyrate is known to be a potent regulator of cell proliferation and gene expression (Gálfi *et al*., 1991, 1993). In contrast, its major catabolite, D-3-hydroxybutyrate, does obviously not modulate proliferative processes (Gálfi *et al*., 1991). Consequently, if metabolism of n-butyrate to D-3-hydroxybutyrate was insufficient, excess n-butyrate could accumulate in the epithelial cell and dysregulate the epithelial cell cycle. It can be speculated that such butyrate effects are involved in the hyper- and parakeratotic alterations of the ruminal epithelium, which are frequently encountered in animals fed high energy diets over a long period, i.e., in chronic acidotic states (Dirksen, 1985,1986; Gäbel, 1990; Owens *et al*., 1998). Energy rich diets do not only lead to a general increase of SCFA production but also to a shift in the molar proportions of the SCFA towards propionate and n-butyrate (Kaufmann *et al*., 1980). The surplus production of n-butyrate may escape epithelial metabolism (Kristensen *et al*., 2000a, b). Thus it is likely that animals with parakeratosis/hyperkeratosis suffer from insufficient local n-butyrate detoxification either due to an increased apical influx of n-butyrate and/or due to lacking adaptation of the intraepithelial metabolism of n-butyrate.

Acid-base balance in the ruminal epithelial cell

The channelling of large amounts of SFCA across the ruminal epithelium represents a constant threat for the intracellular pH of ruminal epithelial cells. This applies both to the diffusive uptake of HSCFA and to the $SCFA^-/HCO_3^-$ exchange (see Figure 1). The diffusive transfer of HSCFA directly decreases the cytosolic pH due to subsequent intracellular release of protons. With $SCFA^-/HCO_3^-$ exchange, on the other hand, there is no direct transfer of protons. However, since HCO_3^- with a pK of 6.1 is replaced by $SCFA^-$ with a pK of 4.8, the intracellular buffer capacity decreases in the physiological range, promoting intracellular acidification indirectly. The partial metabolism of SCFA does not really give relief from this threat as the metabolites are, again, all acids. Consequently, effective mechanisms for alkalisation and regeneration of intracellular buffer capacity are needed to avoid lethal intracellular acidification. Recent functional, molecular and immunocytochemical studies have provided evidence that the pH_i is maintained by various H^+ exporting systems, i.e., Na^+/H^+ exchange (NHE; Müller *et al.*, 2000), a vacuolar H^+-ATPase (Schweigel and Martens, 2003), and a monocarboxylate transporter 1 (MCT1, Müller *et al.*, 2002). The H^+ exporting systems are complemented by at least one HCO_3^- importing system, namely, the sodium bicarbonate cotransporter 1 (NBC1; Huhn *et al.*, 2003). The impact of the various mechanisms for intracellular alkalinization depends on the extracellular conditions and on the localization of the transport proteins. Properties and (assumed) localizations of the pH regulating transport proteins are summarized in Figure 2.

The mentioned transport mechanisms, of course, not only regulate pH disturbances caused by the presence and transport of SCFA. A second major acidifying threat is the bacterial production of huge amounts of CO_2 in the ruminal lumen. On diffusion into the ruminal epithelial cell, protons are released due to conversion of CO_2 into H^+ and HCO_3^-. Therefore, the regulation of intracellular HCO_3^- homeostasis is not only important with respect to SCFA transport but similarly linked to the generation of CO_2 by intraruminal microbes.

The microbes in the rumen can also produce large amounts of ammonia depending on the feeding conditions. Whether ammonia inflow across the cell membrane is an acidifying or an alkalinising factor mainly depends on the form and mechanism of membrane transfer. A predominant uptake of NH_3 and its intracellular protonation would alkalinise the cytoplasm, whereas a predominant uptake of NH_4^+ would acidify the cytoplasm by reversing this reaction. So far, both diffusive uptake of NH_3 and uptake of NH_4^+ have been suggested (Bödecker and Kemkowski, 1996; Abdoun *et al.*, 2003; Martens *et al.* 2004). Studies in cultured ruminal cells indicate a predominance of NH_3 uptake over NH_4^+ uptake, since pH_i shows a rapid and strong raise after exposure of cells to NH_4Cl (Müller *et al.*, 2000). Whether a parallel inflow of NH_3 can contribute *in vivo* to a compensation of the acidifying influx of HSCFA and CO_2 remains to be elucidated.

pH_i regulating mechanisms and extracellular conditions

The ruminal epithelium possesses at least two Na^+-dependent acid/base transporters that counteract pH_i drops during intracellular acid loads. These have been identified as NHE and NBC1 by functional studies in isolated ruminal epithelia and in cultured ruminal epithelial cells and by

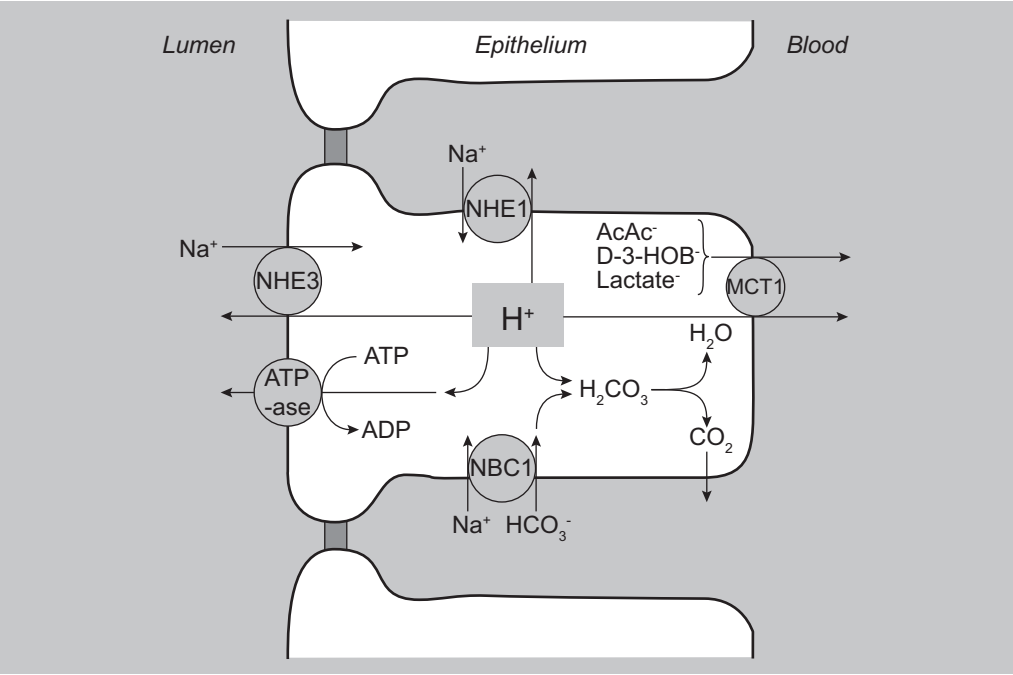

Figure 2. Luminal and systemic release of protons by the ruminal epithelium and restoration of intracellular buffer capacity.
See also legend of Figure 1 for comment on localization of the transport proteins.

molecular biological studies (Gäbel *et al*., 1991b; Sehested *et al*., 1999b; Müller *et al*., 2000; Huhn *et al*., 2003). Both NBC1 and NHE represent important homeostatic mechanisms providing for a neutral pH_i in ruminal epithelial cells. Principally, both transporters help to assure the intactness of the ruminal epithelium in its acidic environment.

At low extracellular HCO_3^- concentrations, NHE seems to be the main mechanism for alkalinization of pH_i (Müller *et al*., 2000; Huhn *et al*., 2003). However, in the presence of HCO_3^-, NHE seems to be of minor importance, since 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), a potent blocker of NHE showed no effect on pH_i regulation in the presence of HCO_3^- (Huhn *et al*., 2003). In contrast, the sodium-coupled HCO_3^- transport (NBC1) seems to be a more important regulator of pH_i in the presence of sufficient extracellular bicarbonate due to effective import of HCO_3^- (Huhn *et al*., 2003; Figure 3).

Besides NHE and NBC1, intracellular alkalinization may theoretically also be achieved by the operation of a vacuolar H^+-ATPase (Schweigel and Martens, 2003). However, the electrogenic H^+-ATPase is probably mainly important for creating an electric driving force across the apical membrane (Schweigel and Martens, 2003). Its quantitative contribution to pH_i regulation remains to be elucidated.

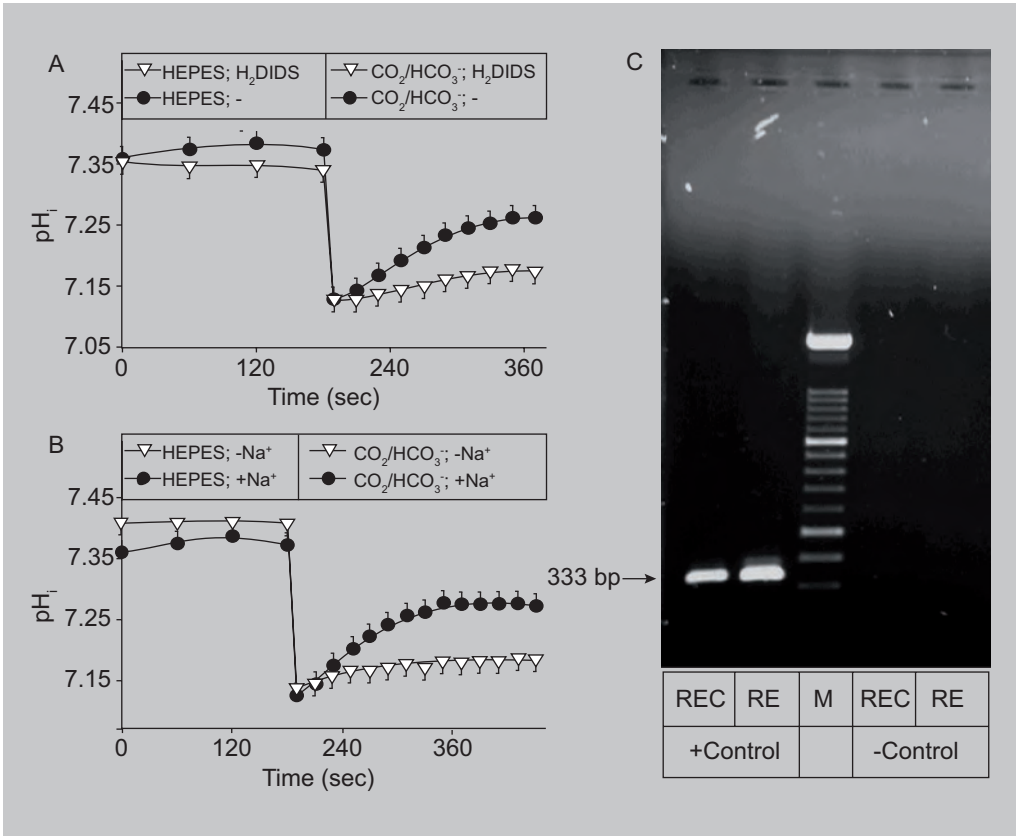

Figure 3. Functional (A/B) and structural evidence (C) for sodium bicarbonate cotransport (NBC1) in the ruminal epithelium.

Functional evidence was achieved by measurement of pH_i recovery from CO_2-induced acid loads in cultured ruminal cells (A/B). pH_i was measured by using the pH-sensitive fluorescent dye BCECF (2´,7´-bis(carboxyethyl)-5(6´)-carboxyfluorescein acetoxymethyl ester). Counter-regulation of pH_i was reduced, when 500 µM H_2DIDS was present in the solutions (A). H_2DIDS is well known to inhibit many bicarbonate transport pathways (Romero and Boron, 1999). Similarly to H_2DIDS, omission of sodium (B) also depressed restoration of pH_i at least, over the time period observed. Apparently, the H_2DIDS-sensitive process displayed an absolute requirement for extracellular Na^+.

Structural evidence (C) was achieved by reverse-transcription (RT)-PCR analysis of sheep ruminal NBC1 by using NBC1 primers derived from the sequence of bovine corneal NBC1. RT-PCR on the near C-terminus region yielded an amplified band of the expected molecular size of 333 bp in both cultured ruminal epithelial cells (REC) and fresh ruminal epithelium (RE). The negative controls contained the appropriate Poly(A)$^+$-RNA instead of cDNA.

Data/Figures modified from Huhn et al. *(2003).*

In addition to NHE, NBC1 and H^+-ATPase, a MCT1 is present which has to be regarded as a more specialized transporter. MCT1 does not only contribute to the elimination of protons. It also mediates the extrusion of acetoacetate, D-3-hydroxybutyrate, and lactate (Müller *et al*., 2002, Figure 4), i.e., the catabolites deriving from the intraepithelial breakdown of SCFA and/or anaerobic glycolysis. D-3-Hydroxybutyric acid, acetoacetic acid, and lactic acid are almost completely dissociated at physiological intracellular pH (~7·37; Müller *et al*., 2000) due to their low pK values (Table 2). These dissociated forms are hardly membrane-permeant. Consequently, without an effective extrusion system, i.e. the MCT1, both the resulting drop in pH_i and intracellular accumulation of the metabolites would indirectly inhibit intracellular metabolism. Additionally, the monocarboxylates are osmotically active, leading to a pathophysiological accumulation of fluid, i.e., cell swelling. Therefore, the extrusion of the monocarboxylates via MCT1 constitutes a prerequisite for: (1) maintaining pH_i homeostasis, (2) intracellular metabolism, and (3) homeostasis of osmolality in ruminal epithelial cells.

Systemic vs. luminal release of protons and acids

Effectivity of pH_i regulating mechanisms does not only depend on external conditions but also on the localization of the transport proteins in the polarized structure of the epithelial cell. The current assumptions and/or knowledge about polarity of acid extruding mechanisms are outlined in Figure 2. Concerning NHE, mRNA for both the NHE3 and the NHE1 isoforms could be detected by molecular biological studies in the ruminal epithelium (Figure 5; Huhn 2004). However, the orientation of the two NHE has not been investigated so far and can only indirectly be derived from functional studies and from findings in other GI-epithelia. In other GI-epithelia, NHE3 is localized on the apical and NHE1 on the basolateral side (Noel and Pouyssegur, 1995; Burckhardt *et al*., 2002). At least, apical NHE activity has been identified in functional studies in isolated ruminal epithelia of sheep and cattle. In these studies, SCFA carried out a strong stimulatory effect on sodium transport from the luminal side to the blood side (Gäbel *et al*., 1991b; Diernaes *et al*., 1994; Sehested *et al*., 1999b; Gäbel *et al*., 1999). The stimulatory effect was explained by the fact that SCFA induce an intracellular acidification, thus increasing the driving force for the apical Na^+/H^+ exchanger (Gäbel and Sehested, 1997).

In agreement with this hypothesis, electroneutral sodium absorption across the ruminal epithelium can be blocked by mucosal (i.e. luminal) application of higher concentrations of the NHE inhibitor, amiloride (Gäbel *et al*., 1991b). If the findings from other epithelia could be transferred directly to the ruminal epithelium, the identity of the apical isoform would likely be NHE3.

Whereas the proposed apical localization of isoform 3 of Na^+/H^+ exchanger seems to be beneficial for the sodium balance of the animal, the correlated proton release into the lumen has to be regarded disadvantageous for the stabilization of pH_i. The protons extruded into the lumen can effectively be recycled, i.e., taken up again, directly by an apical inflow of HSCFA and/or indirectly by inflow of CO_2 after the luminal reaction with HCO_3^- ($H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O$). Therefore, apical NHE (3) cannot be regarded as an effective pH_i regulatory mechanism. Besides the molecular biological studies shown in Figure 5, studies in cultured ruminal epithelial cells also point to the additional presence of NHE1. HOE-694, which is a specific inhibitor of NHE1 isoform

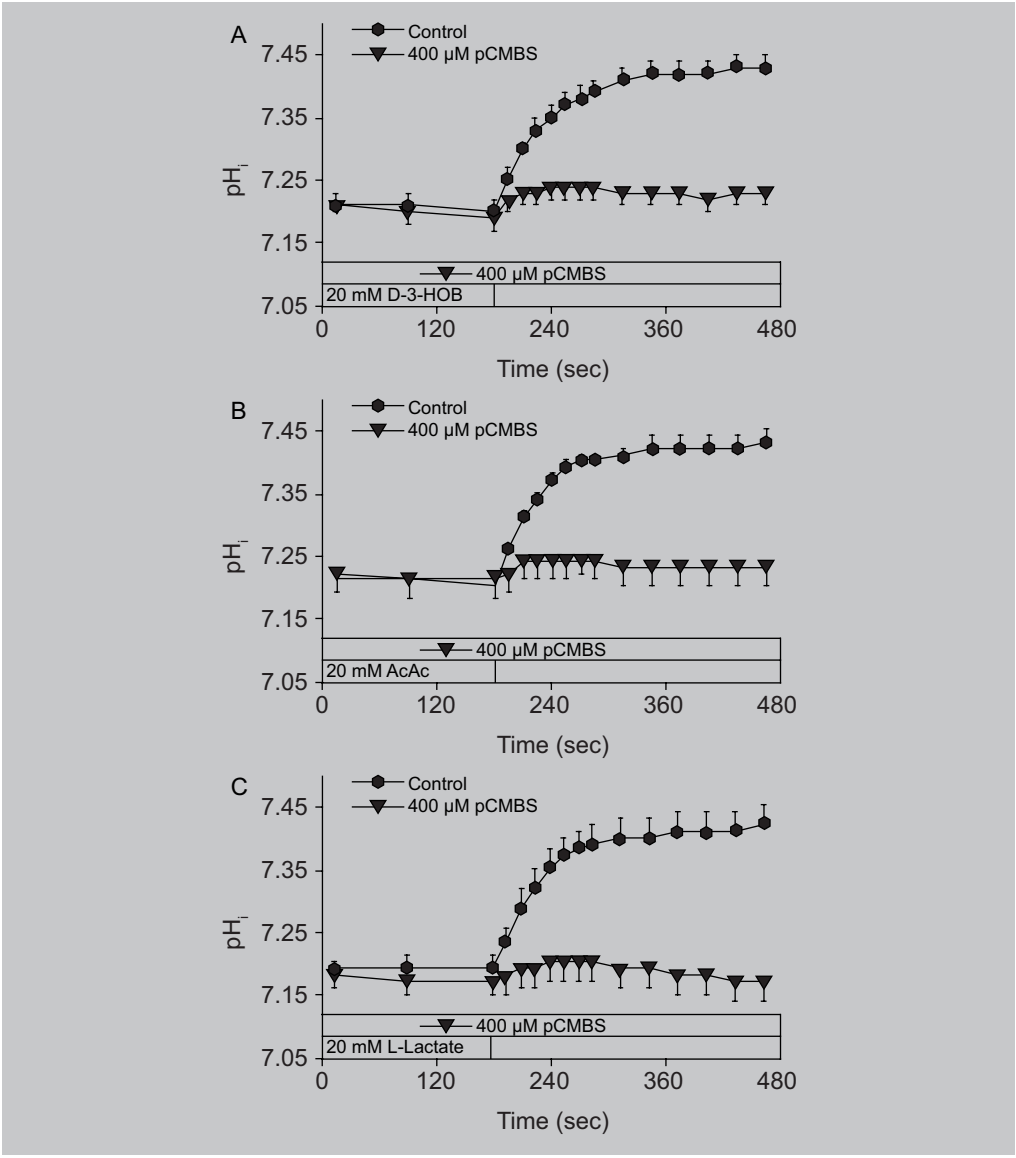

Figure 4. pHi recovery from D-3-HOB-, AcAc- and L-lactate-induced cell acidification in the presence or absence of pCMBS (Source: figure modified from Müller et al.*, 2002).*
Cultured ruminal epithelial cells were pre-incubated for 45 min with D-3-HOB, AcAc or L-lactate (20 mM). During the last three min of incubation, pHi was recorded. Thereafter, cells were switched to D-3-HOB$^-$ (A), AcAc$^-$ (B) and L-lactate$^-$ (C) free standard solution in the presence or absence of 400 µM of p-chloromercuribenzene-sulfonate (pCMBS). pCMBS is a well known inhibitor of MCTs (Carpenter and Halestrap, 1994; Halestrap and Price, 1999; Tosco et al.*, 2000). Removal of extracellular L-lactate, D-3-HOB, or AcAc resulted in a rapid recovery of pH$_i$. pCMBS decreased rate of pH$_i$ recovery by more than 70% indicating that pH$_i$ recovery under these conditions mainly depended on MCT.*

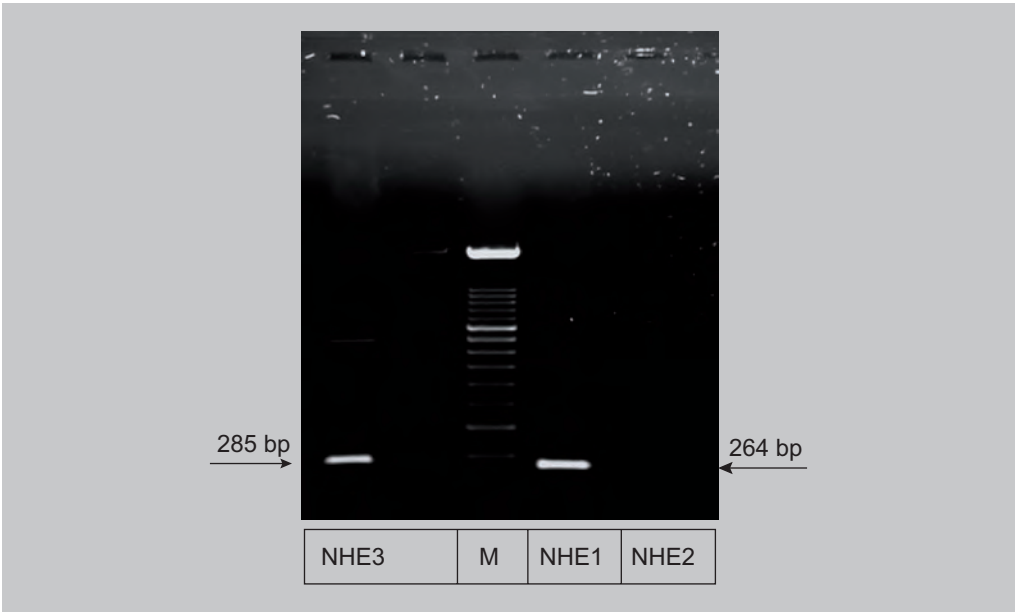

Figure 5. Detection of NHE1 and NHE3 in the sheep rumen epithelium by reverse-transcription (RT)-PCR analysis.
RT-PCR was carried out using specific primers derived from bovine gallbladder epithelium (NHE1; NHE3) and from human colonic epithelium (NHE2). M: DNA size marker. Arrows: Expected molecular sizes of NHE1 and NHE3 fragments.

at low concentrations (Masereel *et al*., 2003), was able to inhibit recovery from intracellular acid load very effectively in cultured ruminal cells (Müller *et al*., 2000). Transferring the results of other GI-epithelia to the rumen, NHE1 should be localized in the basolateral membrane of the ruminal epithelium and has to be regarded as more important for the regulation of pH_i than NHE3.

In contrast to the findings on NHE, the localization of the proton-linked monocarboxylate transporter 1 (MCT1; Müller *et al*., 2002) has been shown by means of immunocytochemical methods. MCT1 seems to be mainly localized on the blood side of the cells in the stratum basale (Figure 6), leading to a preferential extrusion of ketone bodies to the bloodstream. By this, the ketone bodies are delivered to the liver and other organs that are potent to catabolize these substrates. In contrast to the MCT1 expressed in the colon (Cuff *et al*., 2002), however, the monocarboxylate transporter of the ovine ruminal epithelium does not transport short chain fatty acids to an appreciable extent (own unpublished results). Therefore, the extrusion of non-metabolized acids at the basolateral pole is so far not fully clear. Current models assume a predominance of passive diffusion of HSCFA across the basolateral membrane.

The bicarbonate importing system, NBC1, is restricted to the basolateral membrane in nearly all epithelia investigated so far (Jensen *et al*., 1999; Schmitt *et al*., 1999; Thevenod *et al*., 1999).

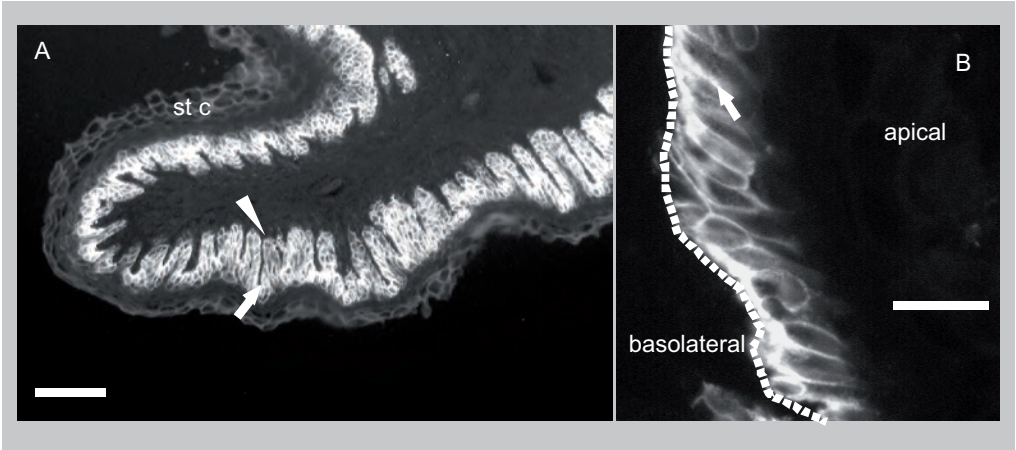

Figure 6. Detection of MCT1 in sheep ruminal epithelium using anti-MCT1 antibodies (Source: modified from Müller et al.*, 2002).*
(A) Arrowhead denotes the basal lamina; st c, the stratum corneum. MCT1 positive cells in the stratum basale of the epithelium are indicated by an arrow. Scale bar represents 100 µm.
(B) MCT1 is primarily expressed in the basolateral membrane of ruminal epithelial cells. Positive staining for MCT1 is pointed out by an arrow. The dotted line indicates localization of the basal lamina. Scale bar represents 25 µm.

A basolateral localization is also probable for NBC1 in the ruminal epithelium as blood with a rather constant HCO_3^- level may serve as a HCO_3^- pool. Using this pool, cells could regulate pH_i efficiently by receiving HCO_3^- out of the blood. Additionally, the blood HCO_3^- pool could provide the cell with HCO_3^- for secretion. Nevertheless, the putative basolateral localization of NBC1 in the ruminal epithelium will have to be verified by immunocytochemical studies.

Functional adaptation of the ruminal epithelium can stabilise the whole system

High yielding ruminants in our current production systems have high energy requirements. For female cattle and sheep, there are additional fluctuations in energy homeostasis according to their reproductive and lactation cycle. These huge and fluctuating energy requirements need to be met by corresponding changes in the energy provision by feeding. The high energy content in ruminant diets often challenges the limits of the microbial ecosystem within the rumen as well as the limits of epithelial absorptive and barrier functions (Gäbel, 1990; Owens *et al*., 1998). The success of energy-dense feeding, therefore, depends closely on whether detrimental pH falls in the ruminal lumen as well as in the ruminal epithelium can be prevented. An adaptation of SCFA channelling across the ruminal epithelium is central in this regard. Adapting ruminants to energy-rich diets can induce an up to four-fold increase in the net absorption/disappearance rate of SCFA (Thorlacius and Lodge, 1973; Dirksen *et al*., 1984; Gäbel *et al*., 1991a; Sehested *et al*., 1997, 2000; Storeheier *et al*., 2003). In parallel, HCO_3^- secretion into the rumen increases (Gäbel *et al*., 1991a). These adaptive changes can be caused by both unspecific and specific mechanisms.

Unspecific adaptive changes of the ruminal epithelium

The key event in elevated transport capacity seems to be morphological transformation of the ruminal epithelium and adjacent tissues. Slowly increasing the concentrate intake leads to increases in the size of papillae, the number of epithelial cells and the number of capillaries (Dirksen *et al*., 1984; Gäbel *et al*., 1987; Pingen, 1990; Shen *et al*., 2002, 2004). Thereby, total absorptive area is increased, epithelial metabolic activity is altered and the drainage of absorbed SCFA is accelerated.

Specific adaptive changes in epithelial transport

The increase of surface area resulting from the proliferative response described above mostly exceeds the increase of absorptive capacity (Gäbel *et al*., 1987; Shen *et al*., 2002). Apart from hyper-/parakeratotic alterations of ruminal epithelium often seen when ruminants are fed high concentrate (pelleted) diets (Kauffold *et al*., 1977; Nocek *et al*., 1984), this could also be linked to a specific down-regulation of transport proteins. By contrast, Sehested *et al.* (2000) revealed an up-regulation of SCFA transport in the absence of morphological changes after a short "SCFA-burst", indicating that SCFA themselves do rather up-regulate the transport proteins involved in absorption. An explanation for these conflicting results is not yet evident. It might be attributable to the fact that different SCFA transport proteins are involved which are not fully elucidated at present. On the other hand, the high baseline absorption of SCFA as described above might complicate detection of down- or up-regulation of a specific SCFA transport protein.

Studies in the colon revealed that MCT1 can be up-regulated by exposing the cells to n-butyrate (Cuff *et al*., 2002). However, these results can hardly be transferred to the ruminal epithelium, since MCT1 is localized on the apical side in the colon (Ritzhaupt *et al*., 1998) and is presumably more important for the transport of SCFA and not of ketone bodies (Ritzhaupt *et al*., 1998; Cuff *et al*., 2002).

Specific adaptations in epithelial metabolism

Like the changes in transport processes, the metabolic processes within the epithelium show also signs of adaptive responses to energy-rich diets. To isolate specific changes in epithelial metabolism from unspecific changes due to morphological adaptation, the metabolic activity needs to be normalised to, e.g., grams of tissue, grams of protein nitrogen or number of cells. When applying these corrections, both up- and down-regulation and also unaffected cell metabolism were reported (Baldwin and McLeod, 2000; Harmon *et al*., 1991; Weigand *et al*., 1975). Taking the total cell mass into account, up-regulation of specific enzymes involved in SCFA degradation was detected (Weekes, 1972).

Regulatory chains of specific and unspecific changes

Various factors induced by energy-rich diets (viscosity and other physical factors, polyamines, nucleotides, specific nutrients, hormones) are discussed to trigger proliferative alterations of gastrointestinal epithelia (Goodlad *et al*., 1991a,b,c; Vandenburgh, 1992; Tanaka *et al*., 1996;

Mathers, 1998; Eliassen and Sjaastad, 2000). Among these factors, n-butyrate probably plays a central role in ruminal epithelium (Sakata, 1995). However, both pro- and antiproliferative effects of n-butyrate are described. The antiproliferative effect is probably due to a direct inhibition of DNA-synthesis by the n-butyrate taken up by the ruminal cell (Gálfi *et al*., 1991; Gálfi *et al*., 1993). The pro-proliferative effect of n-butyrate might be indirect. n-Butyrate has been shown to induce the release of trophic hormones like insulin and growth factors (Jordan and Phillips, 1978; Shen *et al*., 2002), which, in turn, are able to induce proliferation of the ruminal epithelium (Gálfi *et al*., 1991; Gálfi *et al*., 1993; Sakata, 1995). Plasma concentration of insulin-like growth factor-1 (IGF-1) also increases during concentrate feeding in goats (Shen *et al*., 2004), which seems to be independent of concurrent increases in ruminal butyrate production (Nosbush *et al*., 1996). A parallel increase in the IGF-1 receptor (IGF-1R) density in the ruminal epithelium may suggest a stimulating activity of the IGF system on ruminal proliferation and function when feeding energy- and protein-dense diets (Shen *et al*., 2004).

Regarding the specific up-regulation of SCFA-transport proteins, evidence is lacking about the role of nutrients, hormones, growth factors and intracellular messengers that may regulate SCFA-transport of the single cell. The second messenger, cyclic adenosine monophosphate (cAMP), could not be demonstrated to influence ruminal SCFA transport *in vitro*, although sodium absorption was decreased (Gäbel *et al*., 1999). Nevertheless, it is very likely that some kind of hormonal or substrate influence exists on the activity of ruminal SCFA transport proteins. It has been shown for another transport protein, the sodium-dependent ruminal glucose transporter, SGLT-1, that hormonal as well as substrate regulation is generally possible (Aschenbach *et al*., 2002).

Conclusions

In the last years, molecular biological, immunocytochemical and functional studies have provided more and more insight in the mechanisms of ruminal SCFA absorption. Studies on intracellular pH restoration in cultured ruminal epithelial cells gained knowledge both on the regulatory mechanisms of pH_i and SCFA absorption. A plethora of transport proteins like NHE1, NHE3, NBC1, MCT1, AE2, DRA, and PAT1 seems to be directly or indirectly involved in SCFA absorption and/or pH_i regulation. However, the horizontal and vertical organization of the epithelium still awaits clarification. So far, a stratified, multilayered epithelium with both effective protective and absorptive mechanisms seems to be unique. Consequently, the models developed for other GI epithelial can be transferred to the rumen only with great care.

In addition to the open questions regarding organization of the epithelium, its adaptive responses have to be elucidated in more detail. So far, it is known that SCFA elimination and HCO_3^--secretion can be increased by concentrate feeding. However, the triggering substrates/receptors, the systemic influences and the intracellular messenger cascades still await further clarification. Especially the latter seems to be of great importance, since the amount of concentrate fed is still increasing from year to year, leading to the need of livestock with great capabilities to adapt to increasing SCFA production.

Acknowledgements

Studies of the authors presented in the article were supported by the German Research foundation (DFG), the H.W.-Schaumann foundation, the Akademie für Tiergesundheit (AfT), and local foundations. We are grateful to many students working in the lab and to the technicians Petra Philipp, Ines Urbansky and Anke Schmidt-Mähne.

References

Abdoun, K., K. Wolf, G. Arndt & H. Martens (2003). Effect of ammonia on Na+ transport across isolated rumen epithelium of sheep is diet dependent. British Journal of Nutrition, 90, 751-8.

Alper, S.L., R.B. Darman, M.N. Chernova & N.K. Dahl (2002). The AE gene family of Cl/HCO_3^- exchangers. Journal of Nephrology, 15 Suppl 5, S41-53.

Amasaki, H., S. Matsumoto & M. Daigo (1991). Distributions of fibronectin, laminin, type I and IV collagens and carbonic anhydrase isozyme III during bovine ruminal epithelial development. Acta Anatomica Basel, 140, 169-74.

Asari, M., K. Sasaki, Y. Kano & T. Nishita (1989). Immunohistochemical localization of carbonic anhydrase isozymes I, II and III in the bovine salivary glands and stomach. Archives of Histology and Cytology, 52, 337-44.

Aschenbach, J.R., T. Borau & G. Gäbel (2002). Glucose uptake via SGLT-1 is stimulated by beta(2)-adrenoceptors in the ruminal epithelium of sheep. Journal of Nutrition, 132, 1254-7.

Ash, R. & G.D. Baird (1973). Activation of volatile fatty acids in bovine liver and rumen epithelium. Evidence for control by autoregulation. Biochemical Journal, 136, 311-9.

Ash, R.W. & A. Dobson (1963). The effect of absorption on the acidity of rumen contents. Journal of Physiology, 169, 39-61.

Baldwin, R.L. & B.W. Jesse (1992). Developmental changes in glucose and butyrate metabolism by isolated sheep ruminal cells. Journal of Nutrition, 122, 1149-53.

Baldwin, R.L. 6th & K.R. McLeod (2000). Effects of diet forage:concentrate ratio and metabolizable energy intake on isolated rumen epithelial cell metabolism in vitro. Journal of Animal Science, 78, 771-83.

Bergman, E.N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological Reviews, 70, 567-90.

Bergman, E.N. & J.E. Wolff (1971). Metabolism of volatile fatty acids by liver and portal-drained viscera in sheep. American Journal of Physiology, 221, 586-92.

Bilk, S., K. Huhn, K.U. Honscha, H. Pfannkuhe & G. Gäbel (2005). Bicarbonate exporting transporters in the ovine ruminal epithelium. Journal of Comparative Physiology B, 175, 365-74.

Bödeker, D. & J. Kemkowski (1996). Participation of NH4+ in total ammonia absorption across the rumen epithelium of sheep (Ovis aries). Comparative Biochemistry and Physiology A, 114, 305-10.

Britton, R. & C. Krehbiel (1993). Nutrient metabolism by gut tissues. Journal of Dairy Science, 76, 2125-31.

Burckhardt, G., F. Di Sole & C. Helmle-Kolb (2002). The Na^+/H^+ exchanger gene family. Journal of Nephrology, 15 Suppl 5, S3-21.

Carpenter, L. & A.P. Halestrap (1994). The kinetics, substrate and inhibitor specificity of the lactate transporter of Ehrlich-Lettre tumour cells studied with the intracellular pH indicator BCECF. Biochemical Journal, 304 (Pt 3), 751-60.

Carter, M.J. (1971). The carbonic anhydrase in the rumen epithelial tissue of the ox. Biochimica Biophysica Acta, 235, 222-36.

Charney, A.N., L. Micic & R.W. Egnor (1998). Nonionic diffusion of short-chain fatty acids across rat colon. American Journal of Physiology, 274, G518-24.

Cuff, M.A., D.W. Lambert & S.P. Shirazi-Beechey (2002). Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. Journal of Physiology, 539, 361-71.

Diernaes, L., J. Sehested, P.D. Moller & E. Skadhauge (1994). Sodium and chloride transport across the rumen epithelium of cattle in vitro: effect of short-chain fatty acids and amiloride. Experimental Physiology, 79, 755-62.

Dijkstra, J., H. Boer, J. Van Bruchem, M. Bruining & S. Tamminga (1993). Absorption of volatile fatty acids from the rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH and rumen liquid volume. British Journal of Nutrition, 69, 385-96.

Dirksen, G. (1985). Der Pansenazidose-Komplex- neuere Erkenntnisse und Erfahrungen (1). Tierärztliche Praxis, 13, 501-12.

Dirksen, G. (1986). Der Pansenazidose-Komplex- neuere Erkenntnisse und Erfahrungen (2). Tierärztliche Praxis, 14, 23-33.

Dirksen, G., H.G. Liebich, G. Brosi, H. Hagemeister & E. Mayer (1984). Morphologie der Pansenschleimhaut und Fettsäureresorption beim Rind - bedeutende Faktoren für Gesundheit und Leistung. Zentralblatt für Veterinärmedizin A, 31, 414-30.

Eliassen, K.A. & O.V. Sjaastad (2000). Polyamines in the gastro-intestinal tract of goat kids and in the regenerating ruminal epithelium of sheep. Journal of Veterinary Medicine A, 47, 297-310.

Flemstrom, G. (1994). Gastric and duodenal mucosal bicarbonate secretion. In: L.R. Johnson, E.D. Jacobson, J. Christensen, D. Alpers & J.H. Walsh (eds) Physiology of the Gastrointestinal Tract. Raven, New York, 1285pp.

Gäbel, G. (1990). Pansenazidose: Interaktionen zwischen den Veränderungen im Lumen und in der Wand des Pansens. Übersichten der Tierernährung, 18, 1-38.

Gäbel, G., J.R. Aschenbach & F. Muller (2002). Transfer of energy substrates across the ruminal epithelium: implications and limitations. Animal Health Research Reviews, 3, 15-30.

Gäbel, G., M. Bestmann & H. Martens (1991a). Influences of diet, short-chain fatty acids, lactate and chloride on bicarbonate movement across the reticulo-rumen wall of sheep. Journal of Veterinary Medicine A, 38, 523-9.

Gäbel, G., H. Butter & H. Martens (1999). Regulatory role of cAMP in transport of Na+, Cl- and short-chain fatty acids across sheep ruminal epithelium. Experimental Physiology, 84, 333-45.

Gäbel, G. & Martens, H. (1991). Transport of Na and Cl across forestomach epithelium: mechanisms and interactions with short-chain fatty acids. In: T. Tsuda, Y. Sasaki & R. Kawashima (eds) Physiological Aspects of Digestion and Metabolism in Ruminants. Academic Press, Orlando, Florida, 129pp.

Gäbel, G., H. Martens, M. Suendermann & P. Galfi (1987). The effect of diet, intraruminal pH and osmolarity on sodium, chloride and magnesium absorption from the temporarily isolated and washed reticulo-rumen of sheep. Quarterly Journal of Experimental Physiology, 72, 501-11.

Gäbel, G., F. Müller, H. Pfannkuche & J.R. Aschenbach (2001). Influence of isoform and DNP on butyrate transport across the sheep ruminal epithelium. Journal of Comparative Physiology B, 171, 215-21.

Gäbel, G. & J. Sehested (1997). SCFA transport in the forestomach of ruminants. Comparative Biochemistry and Physiology A, 118, 367-74.

Gäbel, G., S. Vogler & H. Martens (1991b). Short-chain fatty acids and CO2 as regulators of Na+ and Cl- absorption in isolated sheep rumen mucosa. Journal of Comparative Physiology B, 161, 419-26.

Gálfi, P., G. Gäbel & H. Martens (1993). Influences of extracellular matrix components on the growth and differentiation of ruminal epithelial cells in primary culture. Research in Veterinary Science, 54, 102-9.

Gálfi, P., Neogrády, S. & Sakata, T. (1991). Effects of volatile fatty acids on the epithelial cell proliferation of the digestive tract and its hormonal mediation. In: T. Tsuda, Y. Sasaki & R. Kawashima (eds) Physiological Aspects of Digestion and Metabolism in Ruminants. Academic Press, San Diego, 50pp.

Giesecke, D., U. Beck & B. Emmanuel (1985). Ketogenic regulation by certain metabolites in rumen epithelium. Comparative Biochemistry and Physiology B, 81, 863-7.

Giesecke, D., U. Beck, S. Wiesmayr & M. Stangassinger (1979). The effect of rumen epithelial development on metabolic activities and ketogenesis by the tissue in vitro. Comparative Biochemistry and Physiology B, 62, 459-63.

Goodlad, R.A., M.A. Ghatei, S.R. Bloom & N.A. Wright (1991a). Glucagon 1-21 Reduces intestinal epithelial cell proliferation in parenterally fed rats. Experimental Physiology, 76, 943-9.

Goodlad, R.A., C.Y. Lee, S. Levin & N.A. Wright (1991b). Effects of the prostaglandin analogue misoprostol on cell proliferation in the canine small intestine. Experimental Physiology, 76, 561-6.

Goodlad, R.A., N. Mandir, S. Levin, J.L. Allen & N.A. Wright (1991c). Prostaglandins and the colonic epithelium - effects of misoprostol on crypt size, cell production, and cell migration in the dog. Gastroenterology, 101, 1229-34.

Halestrap, A.P. & N.T. Price (1999). The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochemical Journal, 343 Pt 2, 281-99.

Han, X.T., P. Noziere, D. Remond, J. Chabrot & M. Doreau (2002). Effects of nutrient supply and dietary bulk on O_2 uptake and nutrient net fluxes across rumen, mesenteric- and portal-drained viscera in ewes. Journal of Animal Science, 80, 1362-74.

Harig, J.M., E.K. Ng, P.K. Dudeja, T.A. Brasitus & K. Ramaswamy (1996). Transport of n-butyrate into human colonic luminal membrane vesicles. American Journal of Physiology, 271, G415-G422.

Harmon, D.L., K.L. Gross, C.R. Krehbiel, K.K. Kreikemeier, M.L. Bauer & R.A. Britton (1991). Influence of dietary forage and energy intake on metabolism and acyl-CoA synthetase activity in bovine ruminal epithelial tissue. Journal of Animal Science, 69, 4117-27.

Holm, M., B. Johansson, A. Pettersson & L. Fandriks (1998). Carbon dioxide mediates duodenal mucosal alkaline secretion in response to luminal acidity in the anesthetized rat. Gastroenterology, 115, 680-5.

Huhn, K. (2004). Regulationsmechanismen des intrazellulären pH-Wertes im Pansenepithel des Schafes. Dissertation, Unversität Leipzig.

Huhn, K., F. Müller, K.U. Honscha, H. Pfannkuche & G. Gäbel (2003). Molecular and functional evidence for a $Na^{(+)}$-$HCO_{(3)}^{(-)}$-cotransporter in sheep ruminal epithelium. Journal of Comparative Physiology B, 173, 277-84.

Huntington, G.B. (1990). Energy metabolism in the digestive tract and liver of cattle: influence of physiological state and nutrition. Reproduction Nutrition Development, 30, 35-47.

Ikuma, M., J. Geibel, H.J. Binder & V.M. Rajendran (2003). Characterization of Cl-HCO_3 exchange in basolateral membrane of rat distal colon. American Journal of Physiology-Cell Physiology, 285, C912-21.

Jacob, P., H. Rossmann, G. Lamprecht, A. Kretz, C. Neff, E. Lin-Wu, M. Gregor, D.A. Groneberg, J. Kere & U. Seidler (2002). Down-regulated in adenoma mediates apical Cl^-/HCO_3^- exchange in rabbit, rat, and human duodenum. Gastroenterology, 122, 709-24.

Jensen, L.J., B.M. Schmitt, U.V. Berger, N.N. Nsumu, W.F. Boron, M.A. Hediger, D. Brown & S. Breton (1999). Localization of sodium bicarbonate cotransporter (NBC) protein and messenger ribonucleic acid in rat epididymis. Biology of Reproduction, 60, 573-9.

Jordan, H.N. & R.W. Phillips (1978). Effect of fatty acids on isolated ovine pancreatic islets. American Journal of Physiology, 234, E162-E167.

Kauffold, P., B. Piatkowski & J. Voigt (1977). Untersuchungen über den Einfluß von Ernährungsfaktoren auf die Pansenschleimhaut. Archiv für Tierernährung, 27, 379-91.

Kaufmann, W., Hagemeister, H. & Dirksen, G. (1980). Adaptation to changes in dietary composition, level and frequency of feeding. In: Y. Ruckebusch & P. Thivend (eds) Digestive Physiology and Metabolism in Ruminants. MTP Press, Lancaster, 587pp.

Kramer, T., T. Michelberger, H. Gürtler & G. Gäbel (1996). Absorption of short-chain fatty acids across ruminal epithelium of sheep. Journal of Comparative Physiology B, 166, 262-9.

Kristensen, N.B., A. Danfaer, V. Tetens & N. Agergaard (1996). Portal recovery of intraruminally infused short-chain fatty acids in sheep. Acta Agricultura Scandinavica, Sect A, Animal Science, 46, 26-38.

Kristensen, N.B., G. Gäbel, S.G. Pierzynowski & A. Danfaer (2000a). Portal recovery of short-chain fatty acids infused into the temporarily- isolated and washed reticulo-rumen of sheep. British Journal of Nutrition, 84, 477-82.

Kristensen, N.B. & D.L. Harmon (2004). Splanchnic metabolism of volatile fatty acids absorbed from the washed reticulorumen of steers. Journal of Animal Science, 82, 2033-42.

Kristensen, N.B., S.G. Pierzynowski & A. Danfaer (2000b). Net portal appearance of volatile fatty acids in sheep intraruminally infused with mixtures of acetate, propionate, isobutyrate, butyrate, and valerate. Journal of Animal Science, 78, 1372-9.

Leo, A., C. Hansch & D. Elkins (1971). Partition coefficients and their uses. Chemical Reviews, 71, 525-616.

Leonhard-Marek, S. (1999). Do forestomach epithelia exhibit a $Mg^{2+}/2H^{+}$-exchanger? Magnesium Research, 12, 99-108.

Lopez, S., F.D. Hovell, J. Dijkstra & J. France (2003). Effects of volatile fatty acid supply on their absorption and on water kinetics in the rumen of sheep sustained by intragastric infusions. Journal of Animal Science, 81, 2609-16.

Martens, H. & G. Gäbel (1988). Transport of Na and Cl across the epithelium of ruminant forestomachs: rumen and omasum. Comparative Biochemistry and Physiology A, 90, 569-75.

Martens, H., T. Krutzfeld & K. Wolf (2004). Sodium transport across the isolated epithelium of sheep omasum is influenced by luminal ammonia. Journal of Veterinary Medicine A, 51, 46-51.

Martens, H. & M. Schweigel (2000). Pathophysiology of grass tetany and other hypomagnesemias. Implications for clinical management. Veterinary Clinics of North America-Food Animal Practice, 16, 339-68.

Masereel, B., L. Pochet & D. Laeckmann (2003). An overview of inhibitors of Na(+)/H(+) exchanger. European Journal of Medicinal Chemistry, 38, 547-54.

Mathers, J.C. (1998). Nutrient regulation of intestinal proliferation and apoptosis. Proceedings of the Nutrition Society, 57, 219-23.

Müller, F., J.R. Aschenbach & G. Gäbel (2000). Role of $Na^{+/}H^{+}$ exchange and HCO_3^- transport in pHi recovery from intracellular acid load in cultured epithelial cells of sheep rumen. Journal of Comparative Physiology B, 170, 337-43.

Müller, F., K. Huber, H. Pfannkuche, J.R. Aschenbach, G. Breves & G. Gäbel (2002). Transport of ketone bodies and lactate in the sheep ruminal epithelium by monocarboxylate transporter 1. American Journal of Physiology-Gastrointestinal and Liver Physiology, 283, G1139-G1146.

Nocek, J.E., C.W. Heald & C.E. Polan (1984). Influence of ration physical form and nitrogen availability on ruminal morphology of growing bull calves. Journal of Dairy Science, 67, 334-43.

Noel, J. & J. Pouyssegur (1995). Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. Americal Journal of Physiology, 268, C283-96.

Nosbush, B.B., Linn J.G., Eisenbeisz W.A., Wheaton J.E. & M.E. White (1996). Effect of concentrate source and amount in diets on plasma hormone concentrations of prepubertal heifers. Journal of Dairy Science, 79, 1400-9.

Noziere, P., C. Martin, D. Remond, N.B. Kristensen, R. Bernard & M. Doreau (2000). Effect of composition of ruminally-infused short-chain fatty acids on net fluxes of nutrients across portal-drained viscera in underfed ewes. British Journal of Nutrition, 83, 521-31.

Ørskov, E.R. (1995). Utilization of short-chain fatty acids in ruminants. In: J.H. Cummings, J.L. Rombeau & T. Sakata (eds) Physiological and Clinical Aspects of Short-Chain Fatty Acids. University Press, Cambridge, 243pp.

Owens, F.N., D.S. Secrist, W.J. Hill & D.R. Gill (1998). Acidosis in cattle: A review. Journal of Animal Science, 76, 275-86.

Peters, J.P., R.Y. Shen & J.A. Robinson (1992). Disappearance of acetic acid from the bovine reticulorumen at basal and elevated concentrations of acetic acid. Journal of Animal Science, 70, 1509-17.

Peters, J.P., R.Y.W. Shen, J.A. Robinson & S.T. Chester (1990). Disappearance and passage of propionic acid from the rumen of the beef steer. Journal of Animal Science, 68, 3337-49.

Pingen, C. (1990). Elektronenmikroskopische Untersuchungen zur fütterungsabhängigen Regression und Proliferation der Pansenschleimhaut beim Rind. Dissertation, Universität München.

Rajendran, V.M. & H.J. Binder (2000). Characterization and molecular localization of anion transporters in colonic epithelial cells. Annals of the New York Academy of Science, 915, 15-29.

Rajendran, V.M., J. Black, T.A. Ardito, P. Sangan, S.L. Alper, C. Schweinfest, M. Kashgarian & H.J. Binder (2000). Regulation of DRA and AE1 in rat colon by dietary Na depletion. American Journal of Physiology-Gastrointestinal and Liver Physiology, 279, G931-42.

Rapoport, S.M. & H.-J. Radebrecht (1977). Physiologisch-Chemisches Praktikum. VEB Volk und Gesundheit, Berlin.

Rechkemmer, G., Gäbel, G., Diernaes, L. *et al.* (1995). Transport of short-chain fatty acids in the forestomach and hindgut. In: W. Engelhardt, S. Leonhard-Marek, G. Breves & D. Giesecke (eds) Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. Ferdinand Enke Verlag, Stuttgart, 93pp.

Rémond, D., J.P. Chaise, E. Delval & C. Poncet (1993). Net flux of metabolites across the ruminal wall of sheep fed twice a day with orchardgrass hay. Journal of Animal Science, 71, 2529-38.

Rémond, D., I. Ortigues & J.-P. Jouany (1995). Energy substrates for the rumen epithelium. Proceedings of the Nutrition Society, 54, 95-105.

Reynolds, D.A., V.M. Rajendran & H.J. Binder (1993). Bicarbonate-stimulated [14C]butyrate uptake in basolateral membrane vesicles of rat distal colon. Gastroenterology, 105, 725-32.

Ritzhaupt, A., I.S. Wood, A. Ellis, K.B. Hosie & S.P. Shirazi-Beechey (1998). Identification and characterization of a monocarboxylate transporter (MCT1) in pig and human colon: its potential to transport L-lactate as well as butyrate. Journal of Physiology (Lond), 513, 719-32.

Robinson, A.M. & D.H. Williamson (1980). Physiological roles of ketone bodies as substrates and signals in mammalian tissues. Physiological Reviews, 60, 143-87.

Romero, M.F. & W.F. Boron (1999). Electrogenic Na^+/HCO_3^- cotransporters: cloning and physiology. Annual Reviews of Physiology, 61, 699-723.

Sakata, T. (1995). Effects of short-chain fatty acids on the proliferation of gut epithelial cells *in vivo*. In J.H. Cummings, J.L. Rumbeau & T. Sakata (eds.) Physiological and Clinical Aspects of Short-Chain Fatty Acids. University Press, Cambridge, 289pp.

Scaife, J.R. & J.Z. Tichivangana (1980). Short chain acyl-CoA synthetases in ovine rumen epithelium. Biochimica et Biophysica Acta, 619, 445-50.

Schmitt B.M., D. Biemesderfer, M.F. Romero, E.L. Boulpaep & W.F. Boron (1999). Immunolocalization of the electrogenic Na^+-HCO^-_3 cotransporter in mammalian and amphibian kidney. American Journal of Physiology, 276, F27-38.

Schweigel, M. & H. Martens (2003). Anion-dependent Mg2+ influx and a role for a vacuolar H+-ATPase in sheep ruminal epithelial cells. American Journal of Physiology-Gastrointestinal and Liver Physiology, 285, G45-53.

Sehested, J., Andersen J.B., O. Aaes, J.B. Kristensen, L. Diernaes, P.D. Moller & E. Skadhauge (2000). Feed-induced changes in the transport of butyrate, sodium and chloride ions across the isolated bovine rumen epithelium. Acta Agricultura Scandinavica, 50, 47-55.

Sehested, J., A. Basse, J.B. Andersen, L. Diernaes, P.D. Moller, E. Skadhauge & O. Aaes (1997). Feed-induced changes in transport across the rumen epithelium. Comparative Biochemistry and Physiology A, 118, 385-6.

Sehested, J., L. Diernaes, P.D. Moller & E. Skadhauge (1999a). Ruminal transport and metabolism of short-chain fatty acids (SCFA) in vitro: effect of SCFA chain length and pH. Comparative Biochemistry and Physiology A, 123, 359-68.

Sehested, J., L. Diernaes, P.D. Moller & E. Skadhauge (1999b). Transport of butyrate across the isolated bovine rumen epithelium-- interaction with sodium, chloride and bicarbonate. Comparative Biochemistry and Physiology A, 123, 399-408.

Sellin, J.H. (1999). SCFAs: The enigma of weak electrolyte transport in the colon. News in Physiological Science, 14, 58-64.

Shen, Z., H.M. Seyfert, B. Lohrke, F. Schneider, R. Zitnan, A. Chudy, S. Kuhla, H.M. Hammon, J.W. Blum, H. Martens, H. Hagemeister & J. Voigt (2004). An energy-rich diet causes rumen papillae proliferation associated with more IGF type 1 receptors and increased plasma IGF-1 concentrations in young goats. Journal of Nutrition, 134, 11-7.

Shen, Z., H.-M. Seyfert, B. Löhrke, F. Schneider, R. Zitnan, A. Chudy, S. Kuhla, H. Hammon, J.W. Blum, H. Martens, H. Hagemeister & J. Voigt (2002). Effects of concentrate intake on rumen papillae development in IGF-1 system in goats. Proceedings of the Society for Nutrition Physiology, 11, 29.

Stevens, C.E. & B.K. Stettler (1966). Factors affecting the transport of volatile fatty acids across rumen epithelium. American Journal of Physiology, 210, 365-72.

Storeheier, P.V. J. Sehested, L. Diernøs, M.A.Sundset, M.A. & S.D. Mathiesen (2003). Effects of seasonal changes in food quality and food intake on the transport of sodium and butyrate across ruminal epithelium of reeindeer. Journal of Comparative Physiology B, 173, 391-99.

Sutherland, T.M. (1963). The metabolism of short chain fatty acids in the ruminant. In: D.P. Cuthbertson (ed) Progress in Nutrition and Allied Sciences. Oliver & Boyd, Edinburgh, 159pp.

Tamminga, S. & A.M. Van Vuuren (1988) Formation and utilization of end products of lignocellulose degradation in ruminants. Animal Feed Science and Technology, 21, 141-59.

Tanaka, M., K. Lee, O. Martinez-Augustin, Y. He, I.R. Sanderson & W.A. Walker (1996). Exogenous nucleotides alter the proliferation differentiation and apoptosis of human small intestinal epithelium. Journal of Nutrition, 126, 424-33.

Thevenod, F., E. Roussa, B.M. Schmitt & M.F. Romero (1999). Cloning and immunolocalization of a rat pancreatic Na(+) bicarbonate cotransporter. Biochemical and Biophysical Research Communications, 264, 291-8.

Thorlacius, S.O. & G.A. Lodge (1973). Absorption of steam-volatile fatty acids from the rumen of the cow as influenced by diet, buffers, and pH. Canadian Journal of Animal Science, 53, 279-88.

Tosco, M., M.N. Orsenigo, G. Gastaldi & A. Faelli (2000). An endogenous monocarboxylate transport in Xenopus laevis oocytes. American Journal of Physiology -Regulatory Integrative and Comparative Physiology, 278, R1190-R1195.

Vandenburgh, H.H. (1992). Mechanical forces and their second messengers in stimulating cell growth in vitro. American Journal of Physiology, 262, R350-5.

Vidyasagar, S, V.M. Rajendran & H.J. Binder (2004). Three distinct mechanisms of HCO3- secretion in rat distal colon. American Journal of Physiology - Cell Physiology, 287, C612-21.

Voet, D. & Voet, J.G. (1992). Lipidstoffwechsel. In: D. Voet & J.G. Voet (eds) Biochemie. VCH, Weinheim, New York, Basel, Cambridge, 619pp.

Walter, A. & J. Gutknecht (1986). Permeability of small nonelectrolytes through lipid bilayer membranes. Journal of Membrane Biology, 90, 207-17.

Wang, Z., S. Petrovic, E. Mann & M. Soleimani (2002). Identification of an apical Cl(-)/HCO3(-) exchanger in the small intestine. American Journal of Physiology -Gastrointestinal and Liver Physiology, 282, G573-9.

Weekes, T.E. (1971). Role of the rumen mucosa in the sheep: relative changes in weight, and in the ability to metabolize propionate, during pregnancy and lactation. Research in Veterinary Science, 12, 373-6.

Weekes, T.E.C. (1972). Effects of pregnancy and lactation in sheep on the metabolism of propionate by the ruminal mucosa and on some enzymic activities in the ruminal mucosa. Journal of Agricultural Science, 79, 409-21.

Weigand, E., J.W. Young & A.D. McGilliard (1975). Volatile fatty acid metabolism by rumen mucosa from cattle fed hay or grain. Journal of Dairy Science, 58, 1294-300.

Part III: Splachnic metabolism

Splanchnic metabolism of long-chain fatty acids in ruminants

J.K. Drackley[1] and J.B. Andersen[2]
[1]University of Illinois, Department of Animal Sciences, Urbana, IL 61801 USA
[2]Danish Institute of Agricultural Sciences, Department of Animal Health, Welfare and Nutrition, Research Centre Foulum, DK-8830 Tjele, Denkmark

Abstract

The liver and portal-drained viscera (PDV) work in series to deliver dietary nutrients and coordinate nutrient use by the rest of the body. The PDV tissues are generally net suppliers of nonesterified fatty acids (NEFA), but may utilize some triacylglycerol (TAG) and NEFA during feed deprivation. The liver takes up more NEFA than the PDV releases, resulting in the total splanchnic tissues being a "sink" for NEFA during mobilization. Production of ketone bodies from NEFA generally is well-matched to NEFA uptake. Hepatic synthesis of very-low-density lipoproteins (VLDL) for export of TAG by nature is not a major fate of NEFA in ruminants. Hepatic lipid accumulation occurs in periparturient dairy cows because of intensive NEFA mobilization. While much has been learned about regulation of hepatic NEFA metabolism, causes of extreme fatty liver and ketosis remain unclear. The capacity of liver tissue to metabolize NEFA via oxidation or esterification changes with physiological state, and may be able to be manipulated to prevent TAG accumulation and to optimize production and health. Strategies to increase oxidative disposal of NEFA may be more successful than attempts to increase VLDL output. Interrelationships of PDV-associated adipose tissues and the liver are fertile areas for future investigation.

Keywords: liver, portal-drained viscera, fatty acids, metabolism, ketone bodies

Introduction

The splanchnic organs include the liver and the portal-drained viscera (PDV), which comprises the stomach, small and large intestines, pancreas, and the mesenteric and omental adipose tissue depots. Because the venous drainage of the PDV flows via the portal vein to the liver, the splanchnic organs function in series to provide the rest of the body with nutrients from both exogenous (dietary) and endogenous sources.

In terms of long-chain fatty acid (LCFA) metabolism, the PDV is responsible for absorption and assimilation of LCFA from the diet and ruminal microorganisms, and distribution of those LCFA as triacylglycerols (TAG) in lipoprotein form to the rest of the body (Noble, 1978; Bauchart, 1993). The PDV also participates in storage of LCFA as TAG in the PDV-associated adipose tissue depots, and the release of LCFA as nonesterified fatty acids (NEFA) that circulate bound to albumin in blood to other tissues of the body. Some tissues of the PDV, particularly the muscle associated with the intestinal tract walls, may oxidize NEFA to obtain a portion of their ATP needs. Moreover, an expanding body of biomedical research indicates that the visceral adipose tissue depots are functionally different from subcutaneous depots, which in turn may impact the liver in both physiological and pathological situations.

The liver plays a central role in coordinating LCFA metabolism in the body. In addition to use of NEFA as a fuel, the liver also converts NEFA taken up from arterial and portal blood to the ketone bodies acetoacetate (AcAc) and ß-hydroxybutyrate (BHBA), particularly in times of starvation or negative energy balance. The liver also re-esterifies NEFA to TAG, which can accumulate in the liver or be packaged as part of very-low-density lipoproteins (VLDL) for secretion and use by peripheral tissues including mammary gland, heart, and skeletal muscle (Bell, 1980; Bauchart, 1993; Grummer, 1993).

During growth and periods of positive nutrient balance, splanchnic metabolism of LCFA plays only a minor role in whole-animal energetics and nutrient utilization. Aspects of LCFA metabolism during growth in ruminants have been reviewed recently (Hocquette and Bauchart, 1999; Drackley, 2005). On the other hand, during times of feed deprivation, late pregnancy, and the transition to lactation, the splanchnic organs play a crucial role in homeostasis and the homeorhetic adaptations to lactation. During this time, TAG accumulates in the liver to varying degress. A considerable body of evidence (see reviews by Rukkwamsuk *et al*., 1999; Katoh, 2002; Jorritsma *et al*., 2003; Bobe *et al*., 2004) implicates accumulation of TAG in the liver as a contributing or predisposing factor to a number of metabolic disorders and infectious diseases in periparturient dairy cows, as well as subsequently impaired reproductive performance and milk production. This chapter will focus primarily on metabolism of LCFA during these times of limiting nutrient availability.

Role of PDV in absorption of dietary LCFA

Although not the primary focus of this chapter, the importance of the PDV tissues in absorption of LCFA of dietary or microbial origin is such that a brief overview of those processes is included here. The LCFA flow from the rumen to the abomasum mostly (ca. 85%) as free (i.e., nonesterified) LCFA adsorbed to particulate matter (Noble, 1978), with the remainder being esterified LCFA primarily in the form of microbial phospholipids. In ruminants, most hydrolysis of complex lipids occurs in the rumen by action of microbial lipases (Harfoot, 1978); however, some dietary TAG may escape the rumen and be digested in the small intestine. Likewise, considerable passage of microbial phospholipids to the duodenum, combined with the influx of phospholipids from bile and sloughed intestinal cells, provides LCFA for absorption after phospholipase-mediated cleavage. Extensive ruminal biohydrogenation of the predominantly unsaturated LCFA consumed in the diet results in the profile of nonesterified LCFA entering the duodenum being primarily saturated. However, addition of unsaturated LCFA as components of biliary phospholipids results in a mixture of saturated and unsaturated LCFA by the time the LCFA reach the jejunum, which is the main site for LCFA absorption (Noble, 1978).

Intestinal absorption of the predominantly saturated LCFA from the rumen is highly efficient (means of 79% and 77% for C16 and C18 fatty acids; Doreau and Chilliard, 1997) as a result of the unique digestive physiology of ruminants. Despite the lack of 2-monoacylglycerols from TAG digestion as occurs in nonruminants, the abundant supply of lecithin (phosphatidylcholine), other phospholipids, and in particular lysolecithin produced by action of phospholipase A2 on lecithin serves to efficiently emulsify the saturated free LCFA into micelles for absorption. Lysolecithin in particular is uniquely well-suited for micelle formation from stearic acid (Freeman, 1969).

After absorption of LCFA into the intestinal epithelial cells and esterification to form acyl-CoA, TAG are reformed through the α-glycerol phosphate pathway and incorporated into TAG-rich lipoproteins (Noble, 1978). In functioning ruminants, these lipoproteins more closely resemble VLDL than the chylomicrons that are produced during digestion of milk lipids by preruminants (Bauchart, 1993). Regardless, the TAG-rich lipoproteins are primarily absorbed into the lymphatic system and thus bypass the liver. Preliminary evidence has been presented that, at least in calves, considerable TAG absorption may occur via the portal vein (see review by Bauchart, 1993, and references therein). However, these observations do not appear to have been confirmed and, except in ruminants consuming very high fat diets, would seem to be of minor importance. Intestinally derived TAG may be the primary source of circulating TAG in ruminants (Emery *et al*., 1992).

Portal absorption of nonesterified LCFA has been observed in nonruminant species (see review by Mu and Hoy, 2004), primarily for medium chain length and unsaturated free LCFA and at high rates of absorption (i.e., large intestinal lipid load). Although not well-studied in ruminants, the relative scarcity of unsaturated LCFA, the essentially constant flow of digesta, and the low lipid content of the diet make the possibility of significant portal absorption unlikely. To the extent that it occurs, portal LCFA absorption would contribute to overestimation of net release, or underestimation of net uptake, of LCFA by the PDV.

Role of splanchnic organs in coordination and use of LCFA

During negative energy balance, adipose depots throughout the body release NEFA into the venous blood as an energy source for other tissues. The concentration of NEFA in peripheral blood is directly proportional to NEFA entry rates (Dunshea *et al*., 1989; Pullen *et al*., 1989), although to our knowledge the extent to which this is true for high NEFA concentrations (in excess of ~500 μ*M*) has not been determined experimentally. The importance of the splanchnic bed in coordinating whole-body use of LCFA is illustrated nicely by data (Table 1) from studies with non-pregnant ewes reported by Heitmann *et al.* (1986). Ewes were studied either in the fed state or after one or three days without feed. Feed deprivation increased arterial NEFA concentration, which was a result of increased net release of NEFA from peripheral adipose depots (represented by hindquarters flux) and PDV adipose depots. Hepatic extraction of NEFA from arterial and portal blood ranged between 15 and 20% of NEFA entering the liver. Liver removal of NEFA was greater than the sum of net PDV and hindquarters NEFA release, such that the total splanchnic bed increased removal of NEFA during starvation. In other words, the total splanchnic tissues were a net "sink" for removal of NEFA from the body.

The fate of NEFA taken up by the liver under these circumstances was primarily conversion to the ketone bodies, AcAc and BHBA. As shown in Table 1, arterial concentrations of both compounds increased during feed deprivation. Flux across the hindquarters was negative and increased after 3 d of starvation, presumably indicating increased uptake and oxidative use of ketone bodies by skeletal muscle of the hindlimb. Flux of both ketone bodies for PDV changed from net output during the fed state, as a result of ruminal production of butyrate and reticuloruminal metabolism to the ketone bodies, to net uptake during the starved state, indicating that arterial-derived ketone bodies now were being used by the PDV tissues. In fed and 1-d starved ewes, the liver removed

Table 1. Net fluxes of nonesterified fatty acids (NEFA), acetoacetate (AcAc), and ß-hydroxybutyrate (BHBA) in splanchnic and lower hindquarters tissues of non-pregnant ewes (Source: adapted from Heitmann et al.*, 1986)*[1].

Variable	Fed	1-d unfed	3-d unfed
NEFA			
Arterial concentration, μ*M*	280	872*	912*
Hindquarters flux, μmol/min	6	nd	92*
PDV flux, μmol/min	63	97	248*
Liver flux, μmol/min	-10	-284*	-407*
Liver extraction, %	15	20	16
Total splanchnic flux[2], μmol/min	-53	-187	-159
AcAc			
Arterial concentration, μ*M*	29.8	35.6	98.1*
Hindquarters flux, μmol/min	-6.3	nd	-15.6*
Hindquarters extraction, %	15	nd	19
PDV flux, μmol/min	45.8	24.7*	-2.2*
Liver flux, μmol/min	-42.7	-35.8	90.0*
Total splanchnic flux[2], μmol/min	3.1	-11.1	87.8
BHBA			
Arterial concentration, μ*M*	549	565	1303
Hindquarters flux, μmol/min	-116	nd	-174
Hindquarters extraction, %	15	nd	14
PDV flux, μmol/min	291	137	-122
Liver flux, μmol/min	276	395	1304
Total splanchnic flux[2], μmol/min	567	532	1182

* Different from fed, $P < 0.05$.

[1] Positive fluxes denote net appearance or production across a tissue bed, negative fluxes denote net disappearance or uptake across a tissue bed. PDV = portal-drained viscera.

[2] Sum of PDV and liver fluxes, calculated from data in paper; not analyzed statistically.

AcAc and converted it to BHBA, but after 3 d of starvation the liver was releasing AcAc on a net basis. Hepatic flux of BHBA was always positive, and increased during starvation. As a result of these changes, total splanchnic flux of AcAc was near zero in fed or 1-d starved ewes but positive in 3-d starved ewes, indicating a switch to net output of this ketone to the rest of the body with advanced starvation. Total splanchnic flux of BHBA was considerable in both fed and 1-d fasted ewes, but about doubled after 3 d of starvation.

Under these conditions, NEFA uptake and ketone body output by the liver were relatively well-matched. Considering the 3-d starved ewes, total ketone body output (AcAc plus BHBA) from the liver was 1394 μmol/min. Total NEFA uptake was 407 μmol/min. If the average NEFA is 17.3 C, NEFA uptake maximally supplied 1760 μmol/min of ketone body precursor. Flux of NEFA to CO_2 in the liver also occurs, but usually is much lower than the conversion to ketone bodies (Jesse *et al.*, 1986a,b; Kleppe *et al.*, 1988). Presumably NEFA uptake in excess of that oxidized

to CO_2 and ketone bodies (and potentially acetate; see later discussion) would be esterified to form TAG. Some of these TAG might be incorporated into hepatic VLDL for secretion from the liver; however, no measurements of TAG metabolism were made in this study. Based on the close agreement between uptake of precursor (NEFA) and output of products (ketone bodies), only a small amount of TAG accumulation would be predicted in the 3-d starved ewes. Indeed, in nonlactating, non-pregnant ruminants, TAG accumulation in the liver during starvation is modest relative to that seen in periparturient or early lactating ruminants (e.g., Lyle *et al*., 1984; Drackley *et al*., 1991b; Grum *et al*., 1994), supporting the implications of the flux data discussed here.

Increased energetic demands during pregnancy and lactation may alter these relationships. Unfortunately, few multi-catheterization studies have been conducted with female ruminants during critical periods of pregnancy and lactation. Freetly and Ferrell (2000) measured splanchnic fluxes of NEFA and TAG in non-pregnant ewes or ewes pregnant with either single or twin lambs. Measurements were made from early pregnancy to near term (6 d before lambing). Arterial NEFA increased as parturition approached (Table 2), with the increases beginning earlier in single-pregnant ewes, and earlier yet for those pregnant with twins. Concentrations at -6 d before lambing were progressively higher with the greater energetic demands of pregnancy. Flux of NEFA from PDV was increased before parturition, but net uptake of NEFA by the liver was much greater. Arterial TAG concentration was not affected by pregnancy in a systematic manner (Table 2); PDV flux of TAG was negative indicating some utilization of TAG by PDV tissues. Liver flux of TAG was usually slightly positive, indicating some net output of TAG from the liver; this output appeared to increase as parturition approached for pregnant lambs. The physiological rationale for this increase is unclear, but could be related to a supply of LCFA for colostrum synthesis in the mammary gland.

At d -6 prepartum, the hepatic TAG output for twin-pregnant ewes was equivalent to 20.5% of net hepatic NEFA uptake. This proportion is similar to estimates (7 to 15%) of the amount of plasma TAG derived from NEFA in dairy cows as determined by isotopic flux measurements in other studies (Palmquist and Conrad, 1971; Palmquist and Mattos, 1978; Pullen *et al*., 1989). In nonruminants, chylomicron synthesis proceeds after a meal, but intestinal synthesis of VLDL appears to continue even during fasting conditions (see review by Phan and Tso, 2001). Although it is possible that a portion of the labeled plasma TAG could have been synthesized in small intestinal cells from arterial NEFA, especially when NEFA concentrations are elevated, data from Pullen *et al.* (1988) showed that, in ewes, blood-derived NEFA contributed very little to intestinally secreted lipoprotein TAG. The nature of the limitations in hepatic VLDL-TAG output is dealt with in a later section.

Changes in splanchnic metabolism of NEFA and TAG in dairy cows across the periparturient period were recently reported by Reynolds *et al.* (2003). Data from that study (Table 3) demonstrate the same patterns of NEFA metabolism by PDV and liver in cows prepartum as discussed already for ewes. Following parturition, arterial NEFA concentration, PDV NEFA output, and hepatic NEFA uptake all were greatest at 11 d postpartum. The total splanchnic tissues again represented a sink for whole-body disposition of NEFA, much of which was converted to ketone bodies (not shown). Arterial TAG concentrations were much lower after parturition, likely due to increased

Table 2. Splanchnic metabolism of nonesterified fatty acids (NEFA) and triacylglycerol (TAG) in non-pregnant and pregnant ewes (adapted from Freetly and Ferrell, 2000)[1].

Variable	No. fetuses	Day relative to parturition				
		-103	-82	-39	-19	-6
NEFA						
Arterial concentration, μ*M*	0	137	147	185	183	203
	1	156	158	206	269	735
	2	139	209	296	756	1162
PDV flux, mmol/h	0	2.96	1.81	1.74	3.06	2.27
	1	1.88	2.22	3.25	3.87	6.08
	2	1.14	3.32	5.91	2.63	5.54
Liver flux, mmol/h	0	-1.04	-2.25	-0.61	-2.58	-3.03
	1	-3.38	-3.48	-3.50	-6.61	-16.54
	2	-2.91	-4.28	-6.63	-15.71	-16.69
TAG						
Arterial concentration, μ*M*	0	107	110	102	135	102
	1	88	95	108	148	78
	2	115	111	175	170	162
PDV flux, mmol/h	0	-1.62	-1.25	-0.26	-1.17	-0.87
	1	-0.80	-0.72	-0.38	-1.11	-0.61
	2	-0.73	-0.77	0.02	-0.39	-0.03
Liver flux, mmol/h	0	0.20	0.17	0.23	0.37	0.32
	1	0.41	0.23	-0.13	1.73	1.14
	2	0.71	0.90	0.11	1.66	1.14

[1] Positive fluxes denote net appearance or production across a tissue bed, negative fluxes denote net disappearance or uptake across a tissue bed. PDV = portal-drained viscera.

removal by the mammary gland for milk fat synthesis. The PDV generally took up small amounts of TAG (with the exception of an apparently aberrant value at -19 d) as seen for ewes. At both 9 d before and 11 d after parturition, liver flux of TAG was negative, indicating a net uptake of TAG rather than output, in contrast to previously discussed data for ewes. The most likely explanation for these findings is an increased clearance of lipoprotein TAG (from remnants of TAG-rich lipoproteins as well as high-density lipoprotein [HDL] clearance), coupled with a low rate of VLDL-TAG output. These more extensive data agree with a limited earlier data set in which feed deprivation resulted in increased net TAG uptake across the liver in two cows (Reid *et al*., 1979).

These data highlight the physiological basis for the commonly observed changes in liver TAG concentrations during the periparturient period in dairy cows (Figure 1). The negative energy balance after parturition, coupled with hormonal changes related to parturition and adaptation to lactation, result in increased lipolysis and decreased re-esterification in adipose tissues. The resultant increases in blood NEFA concentration lead to increased uptake of NEFA by the liver.

Table 3. Splanchnic metabolism of nonesterified fatty acids (NEFA) and triacylglycerols (TAG) in periparturient dairy cows (adapted from Reynolds et al.*, 2003)*[1].

Variable	Day relative to parturition					
	-19	-9	11	21	33	83
NEFA						
Arterial concentration, μ*M*	115	173*	361*	236*	185*	88
PDV flux, mmol/h	16.0	24.0	66.0*	45.0*	36.0	21.0
Liver flux, mmol/h	-21.0	-35.6	-115.8*	-79.9*	-53.5*	-40.2
Liver extraction, %	19.3	22.3	18.3	18.4	16.6	23.0
Splanchnic flux, mmol/h	-5.0	-11.4	-49.8*	-35.5*	-17.5	-18.9
TAG						
Arterial concentration, μ*M*	160	177	39*	55*	65*	71*
PDV flux, mmol/h	16.8	-3.7	0.1	-1.3	-1.0	-1.1
Liver flux, mmol/h	0.7	-9.6*	-2.2	0.0	-1.2	1.2
TG ouput/NEFA uptake, %	10.0	…	…	0.0	…	9.0

* Different from d -19, $P < 0.05$.

[1] Positive fluxes denote net appearance or production across a tissue bed, negative fluxes denote net disappearance or uptake across a tissue bed. PDV = portal-drained viscera.

Data from Reynolds *et al.* (2003) demonstrated that extraction of NEFA from blood is relatively constant (16.6 to 23.0%; Table 3), in agreement with previous observations (summarized by Bell, 1980). Because the liver receives roughly one-third of cardiac output (Huntington *et al.*, 1990), the liver is subjected to a disproportionately high delivery of NEFA relative to its mass. The liver ß-oxidizes much of the NEFA taken up to acetyl-CoA, some of which is oxidized completely to CO_2 in the tricarboxylic acid (TCA) cycle. The remainder of the acetyl-CoA is converted to the ketone bodies or acetate, which serve as important water-soluble fuels that can decrease glucose utilization by many tissues in ruminants, including the heart, kidney, skeletal muscle, mammary gland, and gastrointestinal tract (Heitmann *et al.*, 1987). The remainder of NEFA taken up in excess of demand for, or capacity for, ß-oxidation is esterified to TAG, which is either secreted in the form of VLDL or accumulates in an intracellular lipid droplet.

Secretion of TAG as VLDL vs. TAG accumulation in liver

As indicated previously, hepatic synthesis and secretion of VLDL appears to be a very limited process in ruminants (Emery *et al.*, 1992). Evidence supporting this contention can be found in studies that have used isotopically labeled LCFA (Pullen *et al.*, 1988, 1989), trans-organ balance techniques (Reid *et al.*, 1979; Reynolds *et al.*, 2003), and in vitro systems (Kleppe *et al.*, 1988; Graulet *et al.*, 1998). In the study by Kleppe *et al.* (1988), rates of TAG formation from LCFA by isolated hepatocytes were similar between goats and rats, despite the much higher metabolic rate of the rat cells, but rates of TAG output in lipoproteins were over 20 times lower for the goat cells. Graulet *et al.* (1998) found that TAG accumulation by rat liver slices was 3.4-fold higher than in calf liver slices, but that VLDL-TAG secretion was 6- to 18-fold greater in rats than in calves. Moreover, when hepatic TAG esterification capacity was stimulated in calves by feeding coconut

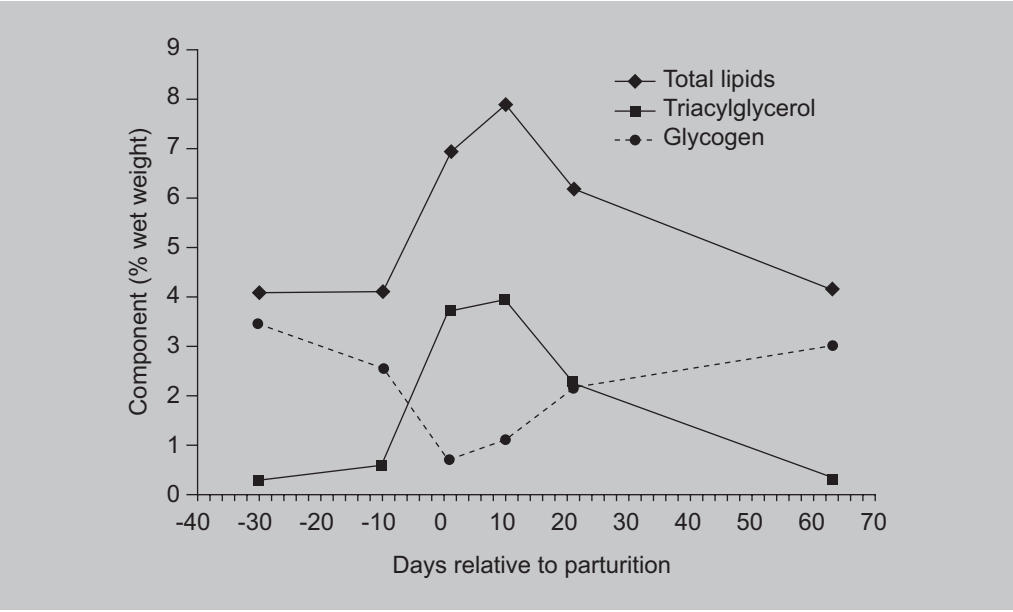

Figure 1. Mean concentrations of total lipid, triacylglycerol, and glycogen in liver tissue from dairy cows (n=60) during the periparturient period (Source: adapted from Underwood, 2003).

oil, output of VLDL-TAG did not increase, in contrast to the effect of dietary coconut oil in rat liver (Graulet *et al*., 2000). Thus, it may be concluded that ruminant liver cells have a similar propensity to esterify LCFA to TAG as do rodents, but less activity in secreting TAG in VLDL.

Considerable research has been conducted to determine the relative expression of various apolipoproteins in the liver of cows that develop fatty livers (e.g., Gruffat *et al*., 1997, and review by Katoh, 2002). Many of the apoproteins, including the primary structural protein of VLDL, apo B100, are decreased during the early post-parturient period and appear to be lower in cows with fatty liver. However, these changes seem to be more a result of fat accumulation rather than a cause of fat infiltration (Katoh, 2002). Indeed, rates of VLDL-TAG output from the liver appear to actually be lower during early lactation (Pullen *et al*., 1989; Reynolds *et al*., 2003) and in the presence of greater hepatic TAG accumulation (Pullen *et al*., 1988; Katoh, 2002). Consequently, the notion that insufficient VLDL synthesis and secretion is an important contributing factor to development of fatty liver is probably not accurate. Whether alterations in lipoprotein metabolism contribute to the downstream effects of fatty liver is not certain but several hypotheses have been advanced (see Katoh *et al*., 2002).

Although Emery *et al.* (1992) stated that "Secretion cannot be an important means of removing fat from ruminant liver", they also suggested that increasing the low capacity for VLDL-TAG output would be a major area of opportunity for preventing or minimizing hepatic TAG accumulation. Since then, considerable research effort has been devoted to understand the mechanisms of limitation and to explore the efficacy of various feed additives or strategies to increase it (see

reviews by Bauchart, 1993; Grummer, 1993; Gruffat *et al*., 1996; Bobe *et al*., 2004; Overton and Waldron, 2004). In particular, the potential for rumen-protected choline products to increase hepatic export of TAG has received considerable recent interest. Because choline is essential for phosphatidylcholine synthesis, which in turn is required for VLDL synthesis in rodent models (Yao and Vance, 1988), and because choline supply in periparturient cows might be suboptimal (Atkins *et al*., 1988), increasing choline supply to the liver might be beneficial. However, no data are available to demonstrate that rumen-protected choline actually increases VLDL-TAG output by the bovine liver. Piepenbrink and Overton (2003) found that increasing doses of rumen-protected choline decreased the incorporation of palmitate into intracellular esterified lipids by liver, with no effect on palmitate oxidation. However, no measurement of VLDL-TAG was made.

Both in vitro and in vivo studies have suggested that newly synthesized TAG enters cytosolic storage pools, but TAG in newly synthesized VLDL originates from a smaller pool within the microsomal compartment (Zammit, 1999a,b; Shelness and Sellers, 2001; Gibbons *et al*., 2004). In rodent models the size of this secretory pool is correlated with the rate of de novo lipogenesis (Gibbons *et al*., 2004). Because hepatic de novo fatty acid synthesis occurs at a neglible rate in ruminants (Pullen *et al*., 1990; Emery *et al*., 1992; Grummer, 1993), it may be logical that size of or flux through the TAG secretory pool also is low. Transfer of TAG from the cytosolic pool to the secretory compartment does not proceed directly because of the limited permeability of TAG in membranes. Instead, cysosolic TAG is hydrolyzed at least to the level of diacylglycerol, which then can be transferred into the microsomal compartment and re-acylated to form TAG for VLDL synthesis (Zammit, 1999a,b; Gibbons *et al*., 2004). The cytosolic TAG pool thus appears to serve as a "buffer" in maintaining a supply of TAG for VLDL synthesis.

The size of the microsomal secretory pool of TAG is much smaller in calf liver than in rat liver (Graulet *et al*., 1998). Rates of apo B100 synthesis were similar between calf and rat liver, but much less apo B was secreted by calf liver slices, suggesting that the majority of newly synthesized apo B was degraded before secretion (Gruffat-Mouty *et al*., 1999). This finding strongly suggests that low plasma or liver concentrations of apo B (Marcos *et al*., 1990; Gruffat *et al*., 1997) are secondary to an as-yet unidentified factor that limits lipid accretion into secretory VLDL. Control of the cytosolic lipase (or lipases) that hydrolyze cytosolic TAG for subsequent translocation into the microsomal compartment, and the intralumenal diacylglycerol acyltransferase (DGAT) enzyme that is responsible for re-synthesis of TAG within the microsomal lumen are likely sites of control that should be investigated in ruminant liver.

Mobilization of cytosolic TAG thus would seem to be a likely site for hormonal control and could be responsible for species differences in hepatic VLDL secretion. Two lipases that may be responsible for the lipolysis of stored cytosolic TAG have recently been identified and characterized in mouse and rat liver. One, called triacylglycerol hydrolase (TGH; Lehner *et al*., 1999) is a microsomal enzyme located close to the cytosolic TAG droplet. Inhibition of TGH activity results in decreased TAG and apo B100 secretion from rat hepatocytes (Gilham *et al*., 2003). Insulin appears to increase re-esterification of the products of TGH activity back to the cytosolic TAG droplet without transfer into the microsomal lumen (Gibbons *et al*., 2004). Of interest also is that synthetic glucocorticoids decrease TGH activity, thereby decreasing TAG

hydrolysis (Dolinsky *et al*., 2004). It is tempting to speculate, therefore, that around parturition the increase in cortisol in dairy cows might suppress activity of TGH, thereby decreasing the apparent secretion of apo B100 (Gruffat *et al*., 1997) and perhaps contributing to the hepatic accumulation of TAG. The second lipase, called arylacetamide deacetylase, also is microsomal in rat liver and its activity is consistent with involvement in VLDL assembly (Trickett *et al*., 2001). Less is known at this point about its regulation.

Exogenous glucagon administration has been shown to lessen TAG accumulation (Hippen *et al*., 1999) and to enhance clearance of accumulated TAG in liver of periparturient dairy cows (Bobe *et al*., 2003). Glucagon had been shown previously to increase clearance of TAG from the liver, although this was originally thought to be due to stimulation of an acid lipase in lysosomes (Debeer *et al*., 1982). The potential impact of glucagon on either of the putative cytosolic TAG lipases would be of interest. Glucagon also may exert stimulatory effects on gluconeogenesis, which may improve hepatic carbohydrate status and, by increasing insulin secretion, modulate the degree of adipose tissue lipolysis and NEFA mobilization.

Gruffat-Mouty *et al.* (1999) suggested that microsomal TAG transfer protein (MTP), which is responsible for transfer of TAG into the growing VLDL particle, might be deficient or inactive in ruminant liver. However, Bremmer *et al.* (1999) reported substantial MTP activity in bovine liver. Furthermore, no relationship existed between measured MTP activity and reported VLDL export rates among a variety of species. In a subsequent study, Bremmer *et al.* (2000) found no relationship between degree of fatty liver and activity of MTP in dairy cows. More recently, Bernabucci *et al.* (2004) showed that mRNA for MTP was actually higher after parturition than before calving in dairy cows.

Control of hepatic ß-oxidation of NEFA

Hepatic oxidative metabolism of LCFA and its regulation in ruminants have been reviewed in detail (Zammit, 1990; Grummer, 1993; Drackley, 1999; Drackley *et al*., 2001) and so will not be exhaustively reviewed here. The liver is supplied with LCFA from uptake of NEFA from blood, from LCFA contained within lipoproteins cleared from the blood, and from the cytosolic TAG droplet discussed above. Following activation of the LCFA to coenzyme-A esters, catalyzed by long-chain acyl-CoA synthetases, the regulation of β-oxidation of LCFA in the liver occurs at several steps. The partitioning of fatty acyl CoA between TAG formation and fatty acyl carnitine esters is the first branch-point within hepatocytes. In rodent liver, the initial steps in esterification and oxidation exist in competition at the outer mitochondrial membrane, catalyzed by the enzymes mitochondrial glycerol-3-phosphate acyltransferase (mGPAT) and carnitine palmitoyltransferase I (CPT I), respectively (Zammit, 1999b). The degree to which this competition occurs in ruminants is uncertain.

In dairy cows that were overfed during the dry period, changes in mGPAT activity did not closely follow the pattern of fatty liver development (Van Den Top *et al*., 1996). Although mGPAT does not appear to be closely correlated with TAG accumulation, from these data it is not possible to definitively conclude that reciprocal regulation of mGPAT and CPT I does not occur in ruminants, because CPT I and other oxidative enzymes were not measured. Data obtained

recently from one of the authors shows that the mRNA for mGPAT increased from d -14 to d 28 relative to parturition, although enzymatic activity was not measured (Loor and Drackley, 2005 unpublished data).

The formation of fatty acyl carnitine from fatty acyl-CoA is catalyzed by CPT I on the outer mitochondrial membrane. Acyl-carnitine translocase then allows entry of the acyl-carnitine into the mitochondria, where CPT II reforms acyl-CoA inside the mitochondria (McGarry and Brown, 1997). Studies with isolated hepatocytes and isolated mitochondria from rat liver have clearly established that CPT I exerts primary flux control on both ß-oxidation and ketogenesis (Drynan *et al*., 1996). Comparable data are not available for ruminants, and current mechanistic models for hepatic metabolism in ruminants do not consider control of LCFA entry into the mitochondria (Hanigan *et al*., 2004). Several studies (Brindle *et al*., 1985; Jesse *et al*., 1986a; Drackley *et al*., 1991a) have provided evidence that CPT I is important for regulating transport of LCFA into the mitochondria for ß-oxidation in ruminants. Activity of CPT I is higher in early lactation than in mid-lactation (Aiello *et al*., 1984). Activity of CPT I is decreased by malonyl-CoA, produced by the enzyme acetyl-CoA carboxylase, in ruminants as in non-ruminants (Brindle *et al*., 1985; Dann, 2004) even though the liver is not a major site of LCFA synthesis in ruminants. As in skeletal muscle of rodents and humans (Ruderman *et al*., 1999; Zammit, 1999b), the presence of acetyl-CoA carboxylase in ruminant liver may be more for purposes of regulating ß-oxidation than for generation of LCFA (Drackley, 1999; Andersen *et al*., 2002b).

Recent studies in one of the authors' laboratories have shown that CPT I activity prepartum is lower in cows that were overfed during the dry period compared with cows that were fed to allow only 80% of energy requirements, although CPT I activity did not differ between groups at d 1 postpartum (Dann, 2004). In rodent liver, the sensitivity of CPT I to inhibition by malonyl-CoA is increased during negative energy balance and vice versa, thereby amplifying the signal (Zammit, 1999b). However, data from dairy cows casts doubt on the importance of this mechanism in periparturient dairy cows (Dann, 2004). Neither activity of CPT I nor its sensitivity to inhibition by malonyl-CoA were related to development of an induced ketosis in dairy cows after parturition (Dann, 2004).

The final steps in regulation of β-oxidation concern the disposal of acetyl-CoA in the mitochondria, which can occur by one of three routes. Acetyl-CoA can be condensed with oxaloacetate to form citrate for complete oxidation to CO_2 in the TCA cycle. Acetyl-CoA can be used to form acetoacetyl-CoA in ketogenesis, with resultant release of acetoacetate that can be interconverted with BHBA by the enzyme BHBA dehydrogenase. Finally, acetyl-CoA can be hydrolyzed by acetyl-CoA hydrolase (Knowles *et al*., 1974), with subsequent release of acetate from the mitochondria.

The rate of ß-oxidation of NEFA as controlled by activity of CPT I presumably is coordinated with other fuels to the need to generate ATP to meet the energetic demands of the liver cell. Conversion of the acetyl-CoA produced by mitochondrial ß-oxidation to ketone bodies allows the liver to ß-oxidize approximately 5 times as much NEFA with the same ATP production as if the acetyl-CoA were completely oxidized in the TCA cycle. Uptake of NEFA in excess of the amount that is able to be ß-oxidized to CO_2, ketone bodies, and acetate is assumed to be esterified. During

negative energy balance, active ß-oxidation of NEFA results in a relatively low intramitochondrial concentration of oxaloacetate as a result of high gluconeogenic flux and the high NADH:NAD^+ ratio in the mitochondria (Zammit, 1990), which also inhibits the dehydrogenase reactions of the TCA cycle (Eaton *et al*., 1994).

Although definitive studies in ruminants are lacking, ketogenesis as a fate for acetyl-CoA produced from ß-oxidation may be regulated by activity of the mitochondrial enzyme HMG-CoA synthase as in other animals. This enzyme is subject to regulation by induction or repression, as well as through auto-succinylation (Hegardt, 1999). In response to depletion of TCA cycle intermediates, indicating a carbohydrate insufficiency, and in the presence of increased acetyl-CoA from ß-oxidation, the enzyme becomes desuccinylated, which increases its activity.

Acetate production from intramitochondrial acetyl-CoA may be another fate of the product of LCFA ß-oxidation and would be a component of the so-called "endogenous" acetate production (Knowles *et al*., 1974). Acetate constituted 16% of the acid-soluble products of radiolabled palmitate metabolism by liver slices (Jesse *et al*., 1986a). Hyrolysis of acetyl-CoA in sheep liver was sufficient to account for rates of hepatic acetate production in vivo (Costa *et al*., 1976). Models of hepatic metabolism in lactating dairy cows suggest that acetate production increases as LCFA supply to the liver is increased (Hanigan *et al*., 2004). In contrast, Pethick *et al.* (1981) concluded that regulation of hepatic acetate production did not correspond to the regulation of ketogenesis. The control and significance of hepatic acetate production in ruminants remain unclear.

Classical respiratory control theory (Brown, 1992) considers that, in general, mitochondrial respiration and, hence, substrate oxidation are controlled by ADP availability that, in turn, is regulated by ATP demands of the cell. Current models for hepatic metabolism in lactating cows suggest that modest increases in availability of NEFA result in coordinate increases in ketone body and acetate output despite predicted decreases in NAD and ADP in mitochondria (Hanigan *et al*., 2004). In highly ketotic states, however, it appears that ß-oxidation of NEFA must be occurring in excess of the ATP demands by the liver. An unresolved question, then, is how this increased ß-oxidation is allowed to continue, presumably no longer constrained by ATP demands. Possible resolutions to this dilemma include 1) greater sparing of ATP generation from other fuels by ß-oxidation of NEFA; 2) an increase in mitochondrial "reverse electron transport" (Berry *et al*., 1983); 3) an induction of uncoupling proteins such as the recently described UCP-2 (Baffy *et al*., 2002) that dissipate heat without ATP formation; or 4) an increase in the proportion of the oxidation that occurs in the peroxisomal ß-oxidation pathway rather than in mitochondria (Grum *et al*., 1994), resulting in less ATP generated per cycle of ß-oxidation. Reverse electron transport as a mechanism to uncouple LCFA oxidation from ATP formation has not been confirmed in ketogenic situations in mammals, and has not been investigated in ruminants. Likewise, the role of alternate uncoupling proteins has not yet been determined for ruminant animals. Neither of these possible mechanisms will be addressed further here.

Relative to the first possibility, the mixture of fuels for ATP production by the liver during different metabolic states remains uncertain. The respiratory quotient across the liver is very low (e.g., 50 to 53%; Reynolds *et al*., 2003), largely as a consequence of the output of incomplete oxidation products such as ketone bodies and acetate rather than CO_2. Metabolic models suggest

that reduced cofactors and ATP are likely not limiting in liver despite a marked deficit in total carbon balance across the liver (see Hanigan *et al*., 2004). Consequently, the extent to which NEFA oxidation continues to replace other fuels as a source of ATP during severe ketosis is not known.

Peroxisomal β-oxidation operates independently of cellular energy status and oxidizes very-long-chain fatty acids and LCFA to produce acetyl-CoA and shortened fatty acids as products (Osmundsen *et al*., 1991). Although peroxisomal oxidation releases energy by oxidation of substrates, the ATP yield is less because the first oxidation step is an oxidase linked to formation of hydrogen peroxide rather than a dehydrogenase linked to an electron transport system as in mitochondrial oxidation. The contribution of peroxisomes to overall fatty acid oxidation in hepatocytes in the basal state remains a subject of debate, but under conditions of impairment or overload of mitochondrial β-oxidation, increased LCFA oxidation via peroxisomal pathways may become more important. Peroxisomal β-oxidation has been reviewed extensively elsewhere (Osmundsen *et al*., 1991; Drackley *et al*., 2001; Van Den Branden and Vameq, 2003; Wanders, 2004).

Hepatic peroxisomal ß-oxidation may contribute considerably to the adaptations of LCFA metabolism in the periparturient period (Grum *et al*., 1994, 1996). In ruminants, the first cycle of peroxisomal β-oxidation accounted for approximately 50% of the total hepatic capacity for first-cycle β-oxidation (Grum *et al*., 1994, 1996; Hansen *et al*., 1995). Peroxisomal β-oxidation in liver homogenates was greater during late pregnancy and early lactation for cows fed a high-fat diet at low dry matter intake during the dry period (Grum *et al*., 1996). That this effect was specific to the endocrine environment around parturition was demonstrated by Grum *et al.* (2002), who found that high-fat diets beginning at 3 wk postpartum did not result in changes in peroxisomal β-oxidation. The in vivo role and potential for manipulation of peroxisomal β-oxidation in periparturient ruminants deserve additional investigation.

Cellular partitioning of hepatic NEFA metabolism

In addition to changes mediated simply by substrate (NEFA) availability, ruminant liver also is subject to changes in NEFA metabolism resulting from enzymatic adaptations according to metabolic state. In particular, metabolic adaptation during the periparturient period in dairy cows (and likely in other ruminant species) involves changes in "specific metabolic capacity", or metabolism per unit of tissue, as a major component (Drackley *et al*., 2001). Liver mass increased by less than 3.5% after parturition compared with prepartum (Reynolds *et al*., 2004); such a small change is not sufficient to account for the marked differences in metabolism that occur following parturition, such as the more than doubling of gluconeogenic rates per unit of liver tissue relative to prepartum values (Drackley *et al*., 2001). Consequently, metabolic adaptation to lactation requires changes in enzymatic activity of hepatic tissue, as reviewed elsewhere (Drackley *et al*., 2001). Here, we highlight some more recent findings and develop the thesis that, because ruminant liver is not designed to secrete large amounts of TAG as VLDL, manipulation of tissue oxidation capacity may be a more attractive approach for minimizing hepatic lipid accumulation and maintaining optimal liver function during the periparturient period.

To determine changes in specific metabolic capacity of liver tissue, we have used a system of liver obtained from dairy cows by biopsy. The liver is either incubated as slices (Grum *et al*., 1996; Litherland *et al*., 2003) or, in the case of small-needle biopsies, the entire bioptate is incubated (Andersen *et al*., 2001). Radiolabeled LCFA, e.g., palmitate, is included in the medium to measure ß-oxidation to CO_2 and to acid-soluble products (ASP), which have been shown to contain predominantly ketone bodies and acetate as well as smaller amounts of labeled TCA cycle and other metabolic intermediates (Jesse *et al*., 1986a). In this way, effects of physiological state or treatments applied to the animal on liver tissue metabolic capacity can be retained in vitro and studied in isolation from confounding effects of differences in substrate supply in the whole-animal. Others have used isolated hepatocytes in suspension culture (Armentano *et al*., 1991) or in longer-term culture systems (Mashek *et al*., 2002; Mashek and Grummer, 2003). However, these systems do not retain the original physiology of the animal and thus are better suited to studying the specific effects of compounds or hormones added in vitro.

In dairy cows studied outside of the periparturient period, physiological state and energy balance affect specific metabolic capacity for NEFA metabolism by liver slices (Drackley *et al*., 1991b; Table 4). As cows progressed to greater energetic demands and increasingly negative energy balance (caused by feed deprivation), the proportion of NEFA oxidized by liver slices (under conditions where substrate was not limiting activity) increased, and the percentage esterified decreased. This type of response might be expected given the greater reliance on NEFA for fuel. In general, liver slices from cows in early lactation have greater rates of ß-oxidation from added NEFA substrate than slices from cows in mid- to late lactation (Aiello *et al*., 1984; Drackley *et al*., 1991a,b).

In contrast, studies with liver obtained from periparturient dairy cows show that capacity for esterification is sharply increased around parturition (Grum *et al*., 1996; Litherland *et al*., 2003), with less of an increase in oxidative capacity. Andersen *et al.* (2002a) found that liver had greater oxidative capacity at 2 wk postpartum compared with 2 wk prepartum, but TAG content was still increased about five-fold (Table 5), suggesting that esterification capacity was not limiting or increased even more. Factors peculiar to the periparturient period may result in a relatively greater esterification capacity in liver tissue during the negative energy balance around parturition than observed at other times of the lactation cycle.

Table 4. Specific metabolic capacity for palmitate in liver slices changes with physiological state of dairy cows (Source: calculated from Drackley et al.*, 1991b).*

Variable	Non-lactating, fed	Lactating, fed	Non-lactating, 7-d starved
Total utilization, nmol/(h x g)	547	477	430
% Oxidized (ASP[1] + CO_2)	23.6	40.7	66.3
% Esterified	76.2	59.3	33.9
[1]Acid-soluble products; includes ketone bodies, acetate, and other intermediates.			

Table 5. In vitro conversion of palmitate to carbon dioxide and acid-soluble products[1] (ASP) by liver biopsies, and contents of triacylglycerol (TAG) and glycogen in liver in periparturient dairy cows (Source: adapted from Andersen et al.*, 2002a).*

Variable	Weeks relative to parturition		
	-2	2	7
CO_2, nmol/(h x g)	4.27	4.07	4.24
ASP, nmol/(h x g)	13.4[a]	17.2[b]	15.7[b]
TAG, μmol/g	3.6[a]	18.9[b]	7.0[a]
Glycogen, μmol/g	264.0[c]	84.1[a]	178.0[b]

[abc], $P < 0.05$ within a row.

[1]Acid-soluble products; includes ketone bodies, acetate, and other intermediates.

Andersen *et al.* (2002a, 2003) studied hepatic specific metabolic capacity in biopsies from dairy cows milked two or three times daily in early lactation and fed either a low or high energy diet (Table 6). Milk production and DMI were greatest for cows fed the high energy diet and milked three times daily (Andersen *et al.*, 2003). Oxidative capacity of liver tissue was greatest for those cows as well, and TAG content of liver tissue was lowest (Andersen *et al.*, 2002a). Cows fed the low-energy diet and milked three times daily were in the poorest energy balance, had the greatest TAG in liver, and had the lowest hepatic capacity to oxidize NEFA. Thus, greater hepatic tissue capacity for NEFA oxidation was associated with greater milk production and less TAG accumulation. The authors (Andersen *et al.*, 2002a) postulated that the increased milk yield and increased intakes of DM and starch resulted in greater gluconeogenesis, which increased ATP demand in hepatocytes and in turn may have stimulated the need for increased ß-oxidation capacity. Increased gluconeogenesis, from both increased substrate supply and the upregulation

Table 6. In vitro conversion of palmitate to carbon dioxide and acid-soluble products (ASP) is inversely related to liver triacylglycerol (TAG) content postpartum and related positively to dry matter intake (DMI) and milk yield (Source: adapted from Andersen et al.*, 2002a, 2003)*

Variable	Dietary energy density and times milked daily[1]			
	L2	L3	H2	H3
DMI[2], kg/d	15.7	16.8	16.9	17.5
Energy intake[2], SFU/d	13.5	14.4	17.9	18.5
Milk yield[3], kg/d	32.0	36.2	39.2	41.1
CO_2, nmol/(h x g)	3.82[ab]	3.23[a]	4.42[b]	5.10[c]
ASP[2], nmol/(h x g)	14.7	13.6	17.3	20.1
TAG[2], μmol/g	12.8	18.5	10.8	10.3
Glycogen, μmol/g	132.1	120.9	132.9	134.3

[abc]Interaction of dietary energy density and milking frequency, $P < 0.05$.

[1]L=low energy density, H=high energy density.

[2]Main effect of dietary energy density, $P < 0.05$. SFU = Scandinavian feed units.

[3]Main effects of dietary energy density and milking frequency, $P < 0.05$.

of gluconeogenic enzymatic capacity (Drackley *et al*., 2001), also could increase the utilization of NADH, which might allow greater LCFA ß-oxidation. This notion is consistent with the idea that, within physiological state, β-oxidation is subject to control by energy status of the hepatocytes as discussed earlier. In agreement with these data, content of TAG in liver at 3 wk postpartum was inversely correlated with both total and peroxisomal ß-oxidation capacity in liver homogenates (Grum *et al*., 2002).

Whether the degree of TAG accumulation in hepatocytes independently affects partitioning of LCFA metabolism is uncertain. Without being able to prove a cause and effect relationship, increased hepatic TAG content in vivo was negatively correlated with in vitro capacity of total and peroxisomal β-oxidation of LCFA (Grum *et al*., 1996). In contrast, increased TAG content of hepatocytes studied in vitro caused an increased capacity for ketone body production (Cadórniga-Valiño *et al*., 1997).

Can hepatic capacity for NEFA ß-oxidation be manipulated?

By extension, then, we propose as others have (Emery *et al*., 1992) that stimulation of hepatic capacity for LCFA ß-oxidation around parturition might lessen TAG accumulation and better maintain hepatic function, which in turn would benefit the animal. If greater oxidative capacity might be beneficial, can it be enhanced through nutritional or pharmacological means?

The effects of increasing L-carnitine supply to cows during negative energy balance have recently been studied (Carlson *et al*., 2004). Although CPT I activity did not appear to be related to TAG accumulation (Dann, 2004), L-carnitine is a second substrate for the CPT I reaction and markedly stimulates LCFA oxidation in vitro (Drackley *et al*., 1991a,b). To establish proof of principle, a large dose (20 g/d) of L-carnitine was infused into the abomasum via a rumen cannula in mid-lactation cows either fed for ad libitum intake or restricted to 50% of ad libitum intake (Carlson *et al*., 2004). In vitro capacity for palmitate oxidation was increased by L-carnitine, and TAG accumulation in vivo during feed restriction was decreased, although TAG concentrations were low as expected in mid-lactation cows. In a second study, increasing doses of L-carnitine were supplied in the diet of cows from 21 d before expected calving to 21 d after calving (Carlson and Drackley, 2005 unpublished). Supplementation of L-carnitine increased oxidative capacity and decreased esterification capacity for palmitate by liver slices in vitro, and decreased in vivo TAG accumulation after parturition in a dose-dependent manner. These studies demonstrate that manipulation of hepatic oxidative capacity for NEFA can impact in vivo accumulation of TAG in the liver of periparturient cows.

Overconditioning and overconsumption of energy during the dry period can impact partitioning of LCFA metabolism in the liver. Liver slices from cows that were overfed, but not overfat, during the dry period had greater esterification capacity and lower oxidation capacity after calving than liver from cows restricted in feed intake (Litherland *et al*., 2003). These findings are in agreement with earlier results of Grum *et al.* (1996). Preliminary evidence indicates that expression of key genes for ß-oxidation, as well as global gene expression profiles in liver also differ substantially between overfed and underfed cows as determined using a bovine-specific cDNA microarray (Loor *et al*., 2004a).

Insulin may be a major physiological regulator of changes in hepatic LCFA partitioning. Through its antilipolytic effects in adipose tissue, increased insulin decreases NEFA supply to the liver (Vernon, 1992; Andersen *et al*., 2002b). In the liver, however, insulin also decreases ß-oxidation capacity (Jesse *et al*., 1986b; Drackley *et al*., 1991a) and increases TAG synthesis (Cadorniga-Valino *et al*., 1997). Andersen *et al.* (2002b) used the hyperinsulinemic, euglycemic clamp technique to determine the effect of increased insulin concentrations on hepatic LCFA oxidative capacity. In both early and mid-lactation cows, insulin decreased incomplete oxidation of palmitate to ASP and tended to decrease complete oxidation to CO_2 (Andersen *et al*., 2002b). If this insulin-mediated suppression occurs before parturition in cows overfed during the dry period, then the sudden increase in NEFA concentration and influx to the liver might overwhelm oxidative capacity and lead to relatively greater TAG formation.

The mechanistic data discussed here are consistent with the many observations that cows which are overfed energy and become overconditioned develop fatty livers around parturition (Morrow, 1976; Fronk *et al*., 1980; Reid *et al*., 1986). Overfeeding and overconditioning have been used experimentally to produce cows that develop fatty livers after parturition (Van Den Top *et al*., 1996; Hippen *et al*., 1999; Murondoti *et al*., 2004). Murondoti *et al.* (2004) found that cows that were overfed prepartum and underfed postpartum developed fatty livers, which were accompanied by decreased activity of 3-hydroxy-acyl-CoA dehydrogenase and unchanged activity of citrate synthase. The authors concluded that prepartum overfeeding, relative to limit feeding to control energy intake, decreased hepatic ß-oxidation of LCFA and thereby contributed to development of fatty liver. However, neither of these enzymes studied is believed to actually limit ß-oxidative flux in vivo. Available data support the description of "type II" ketosis, which develops as cows become more insulin-resistant before parturition due to overfeeding (Holtenius and Holtenius, 1996; Herdt, 2000). Evidence for induction of insulin resistance with prolonged overconsumption of energy by non-lactating cows, even in the absence of overconditioning, has been presented (Holtenius *et al*., 2003; Dann, 2004).

The profile of dietary or mobilized LCFA also could impact hepatic partitioning of LCFA metabolism. As postulated elsewhere, a profile of LCFA presented to the animal that is either markedly more saturated or unsaturated than "normal" for that physiological stage seems to disrupt hepatic metabolism (Drackley, 2005). For example, both coconut oil (high in medium-chain saturated fatty acids; more saturated than milk fat) and soybean oil (high in long-chain unsaturated fatty acids; more unsaturated than milk fat) cause TAG accumulation in liver of calves (Jenkins and Kramer, 1986; Leplaix-Charlat *et al*., 1996; Piot *et al*., 1999; Graulet *et al*., 2000). In monolayer cultures of bovine hepatocytes, docohexanenoic acid (DHA) and eicosapentaenoic acid (EPA) were more readily ß-oxidized than oleic or palmitic acids (Mashek *et al*., 2002). Palmitic and oleic acids were the best substrates for TAG formation, whereas the polyunsaturated LCFA were less incorporated into TAG. Addition of EPA, DHA, or oleic acid increased oxidation of palmitate and polyunsaturated LCFA decreased conversion of palmitate to TAG. In contrast, saturated LCFA stimulated ß-oxidation of palmitate, whereas DHA increased esterification of palmitate to cellular TAG (Mashek and Grummer, 2003).

The enzymes of mitochondrial and peroxisomal ß-oxidation are under transcriptional control through the effects of the nuclear receptor peroxisomal proliferator-activated receptor α (PPARα;

Mandard *et al*., 2004). Activity of this receptor is increased by increased cellular availability of NEFA (Nakamura *et al*., 2004), which bind to and activate PPARα. The activated PPARα dimerizes with the retinoid-X receptor and the dimer binds to specific DNA sequences (response elements) in the promoter region of target genes (Mandard *et al*., 2004). The importance of PPAR to periparturient adaptations in hepatic lipid metabolism has been postulated for some time (Grum *et al*., 1994; Drackley, 1999). The mRNA for PPARα is increased in liver around parturition (Janovick *et al*., 2004), and the pattern of change may be different in cows that are overfed compared with cows fed to requirements or below (Janovick, Loor and Drackley, 2005 unpublished data).

A focus of current research in our laboratories is whether manipulation of PPARα activity by pharmacologic or dietary means may be able to modulate metabolic responses of cows. For example, certain LCFA may be more potent inducers of PPARα than others, and might in part explain results observed in monolayer hepatocyte cultures (Mashek *et al*., 2002; Mashek and Grummer, 2003). Selberg *et al.* (2005) recently reported that feeding fat supplements containing *trans*–octadecenoic acids to periparturient dairy cows upregulated the mRNA for PPARα in liver during the first month of lactation. However, no corresponding effects on TAG accumulation in liver were observed. Potential differential impacts of various LCFA on partitioning of hepatic LCFA metabolism, including the ability to increase ß-oxidation, as well as potential pharmacologic manipulation of PPAR-mediated target genes (Cappon *et al*., 2002) may be fruitful avenues for investigation.

Do PDV adipose tissues influence liver metabolism of LCFA?

As mentioned earlier, the adipose tissue depots of the PDV supply NEFA directly to the liver upon lipolytic stimulation. Whether these internal depots are more or less sensitive to lipolytic and anti-lipolytic stimuli than other adipose depots in ruminants is not clear. It is well-established in other species, however, that visceral adipose depots have different regulatory characteristics (e.g., Monzon *et al*., 2002; Kabir *et al*., 2005) and secrete different profiles of adipokines (Trayhun and Wood, 2004) such as leptin, TNF-α, resistin, and others. Many of these proteins have direct or indirect effects on liver metabolism of NEFA (e.g., Gabriely *et al*., 2002). It is also clear that, in humans, individuals vary substantially in their propensity to deposit fat in subcutaneous or visceral sites (e.g., Gasteyger and Tremblay, 2002) and that adipokine expression varies between these sites (e.g., Dusserre *et al*., 2000). Whether such variation exists in cows fed diets that promote weight gain, so that cows might accrete relatively greater amounts of fat in PDV adipose depots in response to overfeeding, is an attractive hypothesis to investigate. Preliminary data indicate that gene expression profiles differ substantially between internal and subcutaneous adipose tissues in dairy cows, as assessed by a bovine-specific cDNA microarray (Loor *et al*., 2004b).

Likewise, the effects of secretion of bioactive compounds into the portal blood on hepatic metabolism of NEFA should be investigated in periparturient dairy cows. Adipose tissues elaborate and secrete many compounds that have inflammatory properties, such as the cytokines TNF-α, IL-1, and IL6, and secretion of these compounds increases with obesity (Trayhurn and Wood, 2004). Such compounds exert a variety of effects on hepatic metabolism of LCFA in other species (see review by Khovidhunkit *et al*., 2004). In dairy cows, cytokines appear to decrease oxidation

of LCFA as in other species (Waldron *et al*., 2003). The potential interrelationships between the PDV adipose tissues and hepatic metabolism that might affect total splanchnic metabolism of LCFA are important areas for future research. More broadly, because cytokine release also can be stimulated by environmental stressors in addition to infection or trauma around parturition, the cross-talk among the immune system, the neuroendocrine system, and splanchnic metabolism should be a productive area of research that links nutrition and management, as argued elsewhere (Drackley *et al*., 2001).

Conclusions and future perspectives

Together, the available trans-organ balance data and cellular metabolic data provide the following picture of splanchnic LCFA metabolism in ruminant animals. The PDV generally releases NEFA and takes up TAG. The liver takes up an essentially constant proportion of NEFA from blood, resulting in greater NEFA uptake as blood NEFA concentrations increase (during negative energy balance or in response to catecholamine stimulation). The liver ß-oxidizes a portion of NEFA in accordance with its ATP demands, whereas the remaining NEFA is esterified to cellular TAG. While some of this TAG may be subsequently secreted in VLDL, output of VLDL-TAG is low even during intensive lipid mobilization. Consequently, intensive NEFA mobilization leads to TAG accumulation in the liver. Overall, the splanchnic tissues are net utilizers of NEFA and so represent a sink for disposal of NEFA during their mobilization.

Whether this "sink" function is always to the advantage of the animal is debatable. Indeed, the considerable evidence that excessive TAG accumulation in the liver is at the least strongly associated with increased incidence of ketosis and other disorders argues that improved strategies to control fatty liver development are still needed. The available evidence indicates that partitioning of LCFA metabolism between esterification and ß-oxidation may be more easily manipulated than output of VLDL. If the proportion of available LCFA that can be processed through ß-oxidation can be enhanced, thereby decreasing TAG accumulation and improving liver function without decreasing dry matter intake, then health and productive efficiency should be enhanced. Thus, although it seems that there has been scientific focus on splanchnic metabolism of LCFA for decades, the need exists for much more knowledge on regulation of metabolism and cell function. The age of the "omic" technologies (functional genomics, proteomics, metabolomics) offers enhanced tools to determine gene and protein expression patterns and their impacts on metabolite pools in various physiological and pathological states. Coupled with techniques such as trans-organ balance and stable isotope tracer kinetics, the tools of the new biology could bring new understanding of the dynamics and regulation of splanchnic LCFA metabolism in ruminants.

References

Aiello, R.J., T.M. Kenna & J.H. Herbein (1984). Hepatic gluconeogenesis and ketogenic interrelationships in the lactating cow. Journal of Dairy Science, 67, 1707-1715.

Andersen, J.B., N.C. Friggens, K. Sejrsen, M.T. Sorensen, L. Munksgaard & K.L. Ingvartsen (2003). The effects of low vs. high concentrate level in the diet on performance in cows milked two or three times daily in early lactation. Livestock Production Science, 81, 119-128.

Andersen, J.B., T. Larsen & K.L. Ingvartsen (2001). Evaluation of tissue preparation for *in vitro* study of hepatic long-chain fatty acid metabolism. Acta Agriculture Scandinavia, Section A, Animal Science, 51, 47-52.

Andersen, J.B., T. Larsen, M.O. Nielsen & K.L. Ingvartsen (2002a). Effect of energy density in the diet and milking frequency on hepatic long chain fatty acid oxidation in early lactating dairy cows. Journal of Veterinary Medicine A, 49, 177-183.

Andersen, J.B., D.G. Mashek, T. Larsen, M.O. Nielsen & K.L. Ingvartsen (2002b). Effects of hyperinsulinaemia under euglycemic conditions on liver fat metabolism in dairy cows in early and mid-lactation. Journal of Veterinary Medicine A, 49, 65-71.

Armentano, L.E., R.R. Grummer, S.J. Bertics, T.C. Skaar & S.S. Donkin (1991). Effects of energy balance on hepatic capacity for oleate and propionate metabolism and triglyceride secretion. Journal of Dairy Science, 74, 132-139.

Atkins, K.B., R.A. Erdman & J.H. Vandersall (1988). Dietary choline effects on milk yield and duodenal choline flow in dairy cattle. Journal of Dairy Science, 71, 109–116.

Baffy, G., C.Y. Zhang, J.N. Glickman & B.B. Lowell (2002). Obesity-related fatty liver is unchanged in mice deficient for mitochondrial uncoupling protein 2. Hepatology, 35, 753-761.

Bauchart, D. (1993). Lipid absorption and transport in ruminants. Journal of Dairy Science, 76, 3864-3881.

Bell, A.W. (1980). Lipid metabolism in liver and selected tissues and in the whole body of ruminant animals. Progress in Lipid Research, 18, 117-164.

Bernabucci, U., B. Ronchi, L. Basirico, D. Pirazzi, F. Rueca, N. Lacetera & A. Nardone (2004). Abundance of mRNA of apolipoprotein B100, apolipoprotein E, and microsomal triglyceride transfer protein in liver from periparturient dairy cows. Journal of Dairy Science, 87, 2881-2888.

Berry, M.N., D.G. Vlark, A.R. Grivell & P.G. Wallace (1983). The calorigenic nature of hepatic ketogenesis: an explanation for the stimulation of respiration induced by fatty acid substrates. European Journal of Biochemistry, 131, 205-214.

Bobe, G., B.N. Ametaj, J.W. Young & D.C. Beitz (2003). Potential treatment of fatty liver with 14-day subcutaneous injections of glucagon. Journal of Dairy Science, 86, 3138-3147.

Bobe, G., J.W. Young & D.C. Beitz (2004). Pathology, etiology, prevention, and treatment of fatty liver in dairy cows. Journal of Dairy Science, 87, 3105-3124.

Bremmer, D.R., S.J. Bertics, S.A. Besong & R.R. Grummer (2000). Changes in hepatic microsomal triglyceride transfer protein and triglyceride in periparturient dairy cattle. Journal of Dairy Science, 83, 2252-2260.

Bremmer, D.R., S.J. Bertics & R.R. Grummer (1999). Differences in activity of hepatic microsomal triglyceride transfer protein among species. Comparative Biochemistry and Physiology A, 124, 123-131.

Brindle, N.P.J., V.A. Zammit & C.I. Pogson (1985). Regulation of carnitine palmitoyltransferase activity by malonyl-CoA in mitochondria from sheep liver, a tissue with low capacity for fatty acid synthesis. Biochemical Journal, 232, 177-182.

Brown, G.C. (1992). Control of respiration and ATP synthesis in mammalian mitochondria and cells. Biochemical Journal, 284, 1-13.

Cadórniga-Valiño, C., R.R. Grummer, L.E. Armentano, S.S. Donkin & S.J. Bertics (1997). Effects of fatty acids and hormones on fatty acid metabolism and gluconeogenesis in bovine hepatocytes. Journal of Dairy Science, 80, 646-656.

Cappon, G.D., R.C.M. Liu, S.R. Frame & M.E. Hurtt (2002). Effects of the rat hepatic peroxisome proliferator, Wyeth 14,643, on the lactating goat. Drug and Chemical Toxicology, 25, 255-266.

Carlson, D.B., H.M. Dann, N.B. Litherland, J.C. Woodworth & J.K. Drackley (2004). Abomasal infusion of L-carnitine alters hepatic fatty acid metabolism and decreases liver lipid in lactating Holstein cows. Journal of Dairy Science, 87 (Supplement 1), 309. (Abstract)

Costa, N.D., G.H. McIntosh & A.M. Snoswell (1976). Production of endogenous acetate by the liver in lactating ewes. Australian Journal of Biological Sciences, 29, 33-42.

Dann, H.M. (2004). Dietary energy restriction during late gestation in multiparous Holstein cows. Ph.D. dissertation, University of Illinois, Urbana.

Debeer, L.J., A.C. Beynen, G.P. Mannaerts & M.J. Geelen (1982). Lipolysis of hepatic triacylglycerol stores. FEBS Letters, 140, 159-164.

Dolinsky, V.W., D.N. Douglas, R. Lehner & D.E.Vance (2004). Regulation of the enzymes of hepatic microsomal triacylglycerol lipolysis and re-esterification by the glucocorticoid dexamethasone. Biochemical Journal, 378, 967-974.

Doreau, M. & Y. Chilliard (1997). Digestion and metabolism of dietary fat in farm animals. British Journal of Nutrition, 78 (Supplement 1), S15-S35.

Drackley, J.K. (1999). Biology of dairy cows during the transition period: the final frontier? Journal of Dairy Science, 82, 2259-2273.

Drackley, J.K. (2005). Interorgan lipid and fatty acid metabolism in growing ruminants. In: D.G. Burrin and H.J. Mersmann (eds.) Biology of Metabolism in Growing Animals. Elsevier Limited, Edinburgh, UK. pp 323-350.

Drackley, J.K., D.C. Beitz & J.W. Young (1991a). Regulation of in vitro palmitate oxidation in liver from dairy cows in early lactation. Journal of Dairy Science, 74, 1884-1892.

Drackley, J.K., D.C. Beitz & J.W. Young (1991b). Regulation of in vitro metabolism of palmitate by carnitine and propionate in liver from dairy cows. Journal of Dairy Science, 74, 3014-3024.

Drackley, J.K., T.R. Overton & G.N. Douglas (2001). Adaptations of glucose and long-chain fatty acid metabolism in liver of dairy cows during the periparturient period. Journal of Dairy Science, 84 (E Supplement), E100-E112.

Drynan, L., P.A. Quant & V.A. Zammit (1996). Flux control exerted by mitochondrial outer membrane carnitine palmitoyltransferase over ß-oxidation, ketogenesis and tricarboxylic acid cycle activity in hepatocytes isolated from rats in different metabolic states. Biochemical Journal, 317, 791-795.

Dunshea, F.R., A.W. Bell & T.E. Trigg (1989). Relations between plasma non-esterified fatty acid metabolism and body fat mobilization in primiparous lactating goats. British Journal of Nutrition, 62, 51-61.

Dusserre, E., P. Moulin & H. Vidal (2000). Differences in mRNA expression of the proteins secreted by the adipocytes in human subcutaneous and visceral adipose tissues. Biochimica et Biophysica Acta, 1500, 88-96.

Eaton, S., D.M. Turnbull & K. Bartlett (1994). Redox control of ß-oxidation in rat liver mitochondria. European Journal of Biochemistry, 220, 671-681.

Emery, R.S., J.S. Liesman & T.H. Herdt (1992). Metabolism of long chain fatty acids by ruminant liver. Journal of Nutrition, 122, 832-837.

Freeman, C.P. (1969). Properties of fatty acids in dispersions of emulsified lipid and bile salt and the significance of these properties in fat absorption in the pig and the sheep. British Journal of Nutrition, 23, 249-263.

Freetly, H.C. & C.L. Ferrell (2000). Net flux of nonesterified fatty acids, cholesterol, triacylglycerol, and glycerol across the portal-drained viscera and liver of pregnant ewes. Journal of Animal Science, 78, 1380-1388.

Fronk,T.J., L.H. Schultz & A.R. Hardie (1980). Effect of dry period overconditioning on subsequent metabolic disorders and performance of dairy cows. Journal of Dairy Science, 63, 1080-1090.

Gabriely, I., X.H. Ma, X.M. Yang, G. Atzmon, M.W. Rajala, A.H. Berg, P. Scherer, L. Rossetti & N. Barzilai (2002). Removal of visceral fat prevents insulin resistance and glucose intolerance of aging. An adipokine-mediated process? Diabetes, 51, 2951-2958.

Gasteyger, C. & A. Tremblay (2002). Metabolic impact of body fat distribution. Journal of Endocrinological Investigation, 25, 876-883.

Gibbons, G.F., D. Wiggins, A.-M. Brown & A.-M. Hebbachi (2004). Synthesis and function of hepatic very-low-density lipoprotein. Biochemical Society Transactions, 32, 59-64.

Gilham, D., S. Ho, M. Rasouli, P. Martres, D.E. Vance & R. Lehner (2003). Inhibitors of hepatic microsomal triacylglycerol hydrolase decrease very low density lipoprotein secretion. FASEB Journal, 17, 1685-1687.

Graulet, B., D. Gruffat, D. Durand & D. Bauchart (1998). Fatty acid metabolism and very low density lipoprotein secretion in liver slices from rats and preruminant calves. Journal of Biochemistry, 124, 1212-1219.

Graulet, B., D. Gruffat-Mouty, D. Durand & D. Bauchart (2000). Effects of milk diets containing beef tallow or coconut oil on the fatty acid metabolism of liver slices from preruminant calves. British Journal of Nutrition, 84, 309-318.

Gruffat, D., D. Durand, Y. Chilliard, P. Williams & D. Bauchart (1997). Hepatic gene expression of apolipoprotein B100 during early lactation in underfed, high producing dairy cows. Journal of Dairy Science, 80, 657-666.

Gruffat, D., D. Durand, B. Graulet & D. Bauchart (1996). Regulation of VLDL synthesis and secretion in the liver. Reproduction, Nutrition, and Development, 36, 375-389.

Gruffat-Mouty, D., B. Graulet, D. Durand, M.E. Samson-Bouma & D. Bauchart (1999). Apolipoprotein B production and very low density lipoprotein secretion by calf liver slices. Journal of Biochemistry, 126, 188-193.

Grum, D.E., J.K. Drackley & J.H. Clark (2002). Fatty acid metabolism in liver of dairy cows fed supplemental fat and nicotinic acid during an entire lactation. Journal of Dairy Science, 85, 3026-3034.

Grum, D E., J.K. Drackley, R.S. Younker, D.W. LaCount & J.J. Veenhuizen (1996). Nutrition during the dry period and hepatic lipid metabolism of periparturient dairy cows. Journal of Dairy Science, 79, 1850-1864.

Grum, D.E., L.R. Hansen & J.K. Drackley (1994). Peroxisomal ß-oxidation of fatty acids in bovine and rat liver. Comparative Biochemistry and Phyiology, 109B, 281-292.

Grummer, R.R. (1993). Etiology of lipid-related metabolic disorders in periparturient dairy cows. Journal of Dairy Science, 76, 3882-3896.

Hanigan, M.D., L.A. Crompton, C.K. Reynolds, D. Wray-Cahen, M.A. Lomax & J. France (2004). An integrative model of amino acid metabolism in the liver of the lactating dairy cow. Journal of Theoretical Biology, 228, 271-289.

Hansen, L.R., J.K. Drackley, L.L. Berger, D.E. Grum,. J.D. Cremin, Jr., X. Lin & J. Odle (1995). Prenatal androgenization of lambs: II. Metabolism in adipose tissue and liver. Journal of Animal Science, 73, 1701-1712.

Harfoot, C.G. (1978). Lipid metabolism in the rumen. Progress in Lipid Research, 17, 21-54.

Hegardt, F.G. (1999). Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase: a control enzyme in ketogenesis. Biochemical Journal, 338, 569-582.

Heitmann, R.N., D.J. Dawes & S.C. Sensenig (1987). Hepatic ketogenesis and peripheral ketone body utilization in the ruminant. Journal of Nutrition, 117, 1174-1180.

Heitmann, R.N., S.C. Sensenig, C.K. Reynolds, J.M. Fernandez & D.J. Dawes (1986). Changes in energy metabolite and regulatory hormone concentrations and net fluxes across splanchnic and peripheral tissues in fed and progressively fasted ewes. Journal of Nutrition, 116, 2516-2524.

Herdt, T.H. (2000). Ruminant adaptation to negative energy balance. Influences on the etiology of ketosis and fatty liver. Veterinary Clinics of North America, Food Animal Practice, 16, 215-230.

Hippen, A.R., P. She, J.W. Young, D.C. Beitz, G.L. Lindberg, L.F. Richardson & R.W. Tucker (1999). Alleviation of fatty liver in dairy cows with 14-day intravenous infusions of glucagon. Journal of Dairy Science, 82, 1139-1152.

Hocquette, J.F. & D. Bauchart (1999). Intestinal absorption, blood transport and hepatic and muscle metabolism of fatty acids in preruminant and ruminant animals. Reproduction, Nutrition, and Development, 39, 27-48.

Holtenius, K., S. Agenäs, C. Delavaud & Y. Chilliard (2003). Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. Journal of Dairy Science, 86, 883-891.

Holtenius, P. & K. Holtenius (1996). New aspects of ketone bodies in energy metabolism of dairy cows: a review. Journal of Veterinay Medicine A, 43, 579-587.

Huntington, G.B., J.H. Eisemann & J.M. Whitt (1990). Portal blood flow in beef steers: comparison of techniques and relation to hepatic blood flow, cardiac output and oxygen uptake. Journal of Animal Science, 68, 1666-1673.

Janovick, N.A., J.J. Loor, H.M. Dann, H.A. Lewin & J.K. Drackley (2004). Characterization of changes in hepatic expression of inflammation-associated genes during the periparturient period in multiparous Holstein cows using quantitative real time-PCR (RT-PCR). Journal of Dairy Science, 87 (Supplement 1), 460. (Abstract)

Jenkins, K.J. & J.K.G. Kramer (1986). Influence of low linoleic and linolenic acids in milk replacers on calf performance and lipids in blood plasma, heart, and liver. Journal of Dairy Science, 69, 1374-1386.

Jesse, B.W., R.S. Emery & J.W. Thomas (1986a). Control of bovine hepatic fatty acid oxidation. Journal of Dairy Science, 69, 2290-2297.

Jesse, B.W., R.S. Emery & J.W. Thomas (1986b). Aspects of the regulation of long-chain fatty acid oxidation in bovine liver. Journal of Dairy Science, 69, 2298-2303.

Jorritsma, R., T. Wensing, T.A.M. Kruip, P.L.A.M. Vos, and J.P.T.M. Noordhuizen (2003). Metabolic changes in early lactation and impaired reproductive performance in dairy cows. Veterinary Research, 34, 11-26.

Kabir, M., K.J. Catalano, S. Ananthnarayan, S.P. Kim, G.W. Van Citters, M.K. Dea & R.N. Bergman (2005). Molecular evidence supporting the portal theory: a causative link between visceral adiposity and hepatic insulin resistance. American Journal of Physiology, 288, E454-E461.

Katoh, N. (2002). Relevance of apolipoproteins in the development of fatty liver and fatty liver-related peripartum diseases in dairy cows. Journal of Veterinary Medical Science, 64, 293-307.

Khovidhunkit, W., M.S. Kim, R.A. Memon, J.K. Shigenaga, A.H. Moser, K.R. Feingold & C. Grunfeld (2004). Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. Journal of Lipid Research, 45, 1169-1196.

Kleppe, B.B., R.J. Aiello, R.R. Grummer & L.E. Armentano (1988). Triglyceride accumulation and very low density lipoprotein secretion by rat and goat hepatocytes in vitro. Journal of Dairy Science, 71, 1813-1822.

Knowles, S.E., I.G. Jarrett, O.W. Filsell & F.J. Ballard (1974). Production and utilization of acetate in mammals. Biochemical Journal, 142, 401-411.

Lehner, R., Z. Cui & D.E. Vance (1999). Subcellular localization, developmental expression and characterization of a liver triacylglycerol hydrolase. Biochemical Journal, 338, 761-768.

Leplaix-Charlat, L., D. Durand & D. Bauchart (1996). Effects of diets containing tallow and soybean oil with or without cholesterol on hepatic metabolism of lipids and lipoproteins in the preruminant calf. Journal of Dairy Science, 79, 1826-1835.

Litherland, N.B., H.M. Dann, A.S. Hansen & J.K. Drackley (2003). Prepartum nutrient intake alters metabolism by liver slices from peripartal dairy cows. Journal of Dairy Science, 86 (Supplement 1), 105-106. (Abstract)

Loor, J.J., N.A. Janovick, H.M. Dann, R.E. Everts, S.L. Rodriguez-Zas, H.A. Lewin, and J.K. Drackley (2004a). Microarray analysis of hepatic gene expression from dry-off through early lactation in dairy cows fed at two intakes during the dry period. Journal of Dairy Science, 87 (Supplement 1), 196. (Abstract)

Loor, J.J., N.A. Janovick, R.E. Everts, S.L. Rodriguez-Zas, H.A. Lewin & J.K. Drackley (2004b). Adipose, mammary, and hepatic gene expression profiling in lactating dairy cows using a bovine cDNA microarray. Journal of Dairy Science, 87 (Supplement 1), 196-197. (Abstract)

Lyle, R.R., G. deBoer, R.O. Harrison & J.W. Young (1984). Plasma and liver metabolites and glucose kinetics as affected by prolonged ketonemia-glucosuria and fasting in steers. Journal of Dairy Science, 67, 2274-2282.

Mandard, S., M. Muller & S. Kersten (2004). Peroxisome proliferator-activated receptor α target genes. Cellular and Molecular Life Sciences, 61, 393-416.

Marcos, E., A. Mazur, P. Cardot & Y. Rayssiguier (1990). Serum apolipoproteins B and A-I and naturally occurring fatty liver in dairy cows. Lipids, 25, 575-577.

Mashek, D.G., S.J. Bertics & R.R. Grummer (2002). Metabolic fate of long-chain unsaturated fatty acids and their effects on palmitic acid metabolism and gluconeogenesis in bovine hepatocytes. Journal of Dairy Science, 85, 2283-2289.

Mashek, D.G., and R.R. Grummer (2003). Effects of long chain fatty acids on lipid and glucose metabolism in monolayer cultures of bovine hepatocytes. Journal of Dairy Science, 86, 2390-2396.

McGarry, J.D. & N.F. Brown (1997). The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. European Journal of Biochemisty, 244, 1-14.

Monzon, J.R., R. Basile, S. Heneghan, V. Udupi & A. Green (2002). Lipolysis in adipocytes isolated from deep and superficial subcutaneous adipose tissue. Obesity Research, 10, 266-269.

Morrow, D.A. (1976). Fat cow syndrome. Journal of Dairy Science, 59, 1625-1629.

Mu, H. & C.E. Hoy (2004). The digestion of dietary triacylglycerols. Progress in Lipid Research, 43, 105-133.

Murondoti, A., R. Jorritsma, A.C. Beynen, T. Wensing & M.J. Geelen (2004). Unrestricted feed intake during the dry period impairs the postpartum oxidation and synthesis of fatty acids in the liver of dairy cows. Journal of Dairy Science, 87, 672-679.

Nakamura, M.T., Y. Cheon, Y. Li & T.Y. Nara (2004). Mechanisms of regulation of gene expression by fatty acids. Lipids, 39, 1077-1083.

Noble, R.C. (1978). Digestion, absorption and transport of lipids in ruminant animals. Progress in Lipid Research, 17, 55-91.

Osmundsen, H.J., J. Bremer & J.I. Pedersen (1991). Metabolic aspects of peroxisomal beta-oxidation. Biochimica et Biophysica Acta, 1085, 141-158.

Overton, T.R. & M.R. Waldron (2004). Nutritional management of transition dairy cows: strategies to optimize metabolic health. Journal of Dairy Science, 87, E105-E119.

Palmquist, D.L. & H.R. Conrad (1971). Origin of plasma fatty acids in lactating cows fed high grain or high fat diets. Journal of Dairy Science, 54, 1025-1033.

Palmquist, D.L. & W. Mattos (1978). Turnover of lipoproteins and transfer to milk fat of dietary (1-carbon-14) linoleic acid in lactating cows. Journal of Dairy Science, 61, 561-565.

Pethick, D.W., D.B. Lindsay, P.J. Barker & A.J. Northrop (1981). Acetate supply and utilization by the tissues of sheep in vivo. British Journal of Nutrition, 46, 97-110.

Phan, C.T., and P. Tso (2001). Intestinal lipid absorption and transport. Frontiers in Bioscience, 6, 299-319.

Piepenbrink, M.S. & T.R. Overton (2003). Liver metabolism and production of cows fed increasing amounts of rumen-protected choline during the periparturient period. Journal of Dairy Science, 86:1722-1733.

Piot, C., J.F. Hocquette, J.H. Veerkamp, D. Durand & D. Bauchart (1999). Effects of dietary coconut oil on fatty acid oxidative capacity of the liver, the heart, and skeletal muscles in the preruminant calf. British Journal of Nutrition, 82, 299-308.

Pullen, D.L., R.S. Emery & N.K. Ames (1988). Turnover of hepatic and plasma triacylglycerol in sheep. Journal of Animal Science, 66, 1538-1547.

Pullen, D.L., J.S. Liesman & R.S. Emery (1990). A species comparison of liver slice synthesis and secretion of triacylglycerol from nonesterified fatty acids in media. Journal of Animal Science, 68, 1395-1399.

Pullen, D.L., D.L. Palmquist & R.S. Emery (1989). Effect of days of lactation and methionine hydroxyl analog on incorporation of plasma fatty acids into plasma triglycerides. Journal of Dairy Science, 72, 49-58.

Reid, I.M., R.A. Collins, G.D. Baird, C.J. Roberts & H.W. Symonds (1979). Lipid production rates and pathogenesis of fatty liver in fasted cows. Journal of Agricultural Science (Cambridge), 93, 253-256.

Reid, I.M., C.J. Roberts, R.J. Treacher & L.A. Williams (1986). Effect of body condition at calving on tissue mobilization, development of fatty liver and blood chemistry of dairy cows. Animal Production, 43, 7-15.

Reynolds, C.K., P.C. Aikman, B. Lupoli, D.J. Humphries & D.E. Beever (2003). Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. Journal of Dairy Science, 86, 1201-1217.

Reynolds, C.K., B. Durst, B. Lupoli, D.J. Humphries & D.E. Beever (2004). Visceral tissue mass and rumen volume in dairy cows during the transition from late gestation to early lactation. Journal of Dairy Science, 87, 961-971.

Ruderman, N.B., A.K. Saha, D. Vavvas & L.A. Witters (1999). Malonyl-CoA, fuel sensing, and insulin resistance. American Journal of Physiology, 276, E1-E18.

Rukkwamsuk, T., T.A. Kruip & T. Wensing (1999). Relationship between overfeeding and overconditioning in the dry period and the problems of high producing dairy cows during the postparturient period. Veterinary Quarterly, 21, 71-77.

Selberg, K.T., C.R. Staples, N.D. Luchini & L. Badinga (2005). Dietary *trans* octadecenoic acids upregulate the liver gene encoding peroxisome proliferator-activated receptor-α in transition dairy cows. Journal of Dairy Research, 72, 107-114.

Shelness, G.S. & J.A. Sellers (2001) Very-low-density lipoprotein assembly and secretion. Current Opinion in Lipidology, 12, 151-157.

Trayhurn, P. & I.S. Wood (2004). Adipokines: inflammation and the pleiotropic role of white adipose tissue. British Journal of Nutrition, 92, 347-355.

Trickett, J.I, D.D. Patel, B.L. Knight, E.D. Saggerson, G.F. Gibbons & R.J. Pease (2001). Characterization of the rodent genes for arylacetamide deacetylase, a putative microsomal lipase, and evidence for transcriptional regulation. Journal of Biological Chemistry, 276, 39533-39532.

Underwood, J.P. (2003). Protein nutrition of dairy cows during the transition period. Ph.D. dissertation, University of Illinois, Urbana.

Van Den Branden, C. & J. Vamecq (2003). Metabolic regulation of peroxisomal and mitochondrial fatty acid oxidation. Advances in Experimental Medicine and Biology, 544, 307-314.

Van Den Top, A.M., M.J.H. Geelen, T. Wensing, G.H. Wentink, A.T. Van 't Klooster & A.C. Beynen (1996). Higher postpartum hepatic triacylglycerol concentrations in dairy cows with free rather than restricted access to feed during the dry period are associated with lower activities of hepatic glycerolphosphate acyltransferase. Journal of Nutrition, 126, 76-85.

Vernon, R.G. (1992). Effects of diet on lipolysis and its regulation. Proceedings of the Nutrition Society, 51, 397-408.

Waldron, M.R., T. Nishida, B.J. Nonnecke & T.R. Overton (2003). Effect of lipopolysaccharide on indices of peripheral and hepatic metabolism in lactating cows. Journal of Dairy Science, 86, 3447-3459.

Wanders, R.J. (2004). Peroxisomes, lipid metabolism, and peroxisomal disorders. Molecular Genetics and Metabolism, 83, 16-27.

Yao, Z. & D.E. Vance (1988). The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. Journal of Biological Chemistry, 263, 2998–3004.

Zammit, V.A. (1990). Ketogenesis in the liver of ruminants — adaptations to a challenge. Journal of Agricultural Science (Cambridge), 115, 155-162.

Zammit, V.A. (1999a). Carnitine acyltransferases: functional significance of subcellular distribution and membrane topology. Progress in Lipid Research, 38, 199-224.

Zammit, V.A. (1999b). The malonyl-CoA-long-chain acyl-CoA axis in the maintenance of mammalian cell function. Biochemical Journal, 343, 505-515.

Splanchnic amino acid metabolism in ruminants

C.K. Reynolds
Department of Animal Sciences, The Ohio State University, OARDC, 1680 Madison Avenue, Wooster, Ohio, 44691-4076, USA.

Abstract

The portal-drained viscera (PDV) and liver, together called the total splanchnic tissues, are extremely active metabolically, accounting for as much as 50% of body oxygen use, and have a high rate of protein synthesis. As a consequence of their many important functions the splanchnic tissues have a large requirement for amino acids, which reduces the net availability of amino acids for other productive purposes. Comparison of measurements of amino acid flux obtained using gut cannulation and multicatheterization techniques suggest that the extensive metabolism of the splanchnic tissues limits amino acid availability to peripheral tissues. This is often presumed to be due to metabolism during the 'first-pass' of amino acids through the absorptive tissues and liver. However, data from studies using isotopically labeled amino acids have shown that most essential amino acids utilized by the PDV are derived from arterial blood, thus the PDV imposes less restriction on entry into blood than suggested by net flux measurements. There is no question that the liver is an important site of amino acid catabolism, but the majority of the amino acids supplied to the liver are also derived from the arterial blood pool. Therefore, the metabolism of amino acids by both the PDV and liver is sensitive to variations in the supply of metabolizable protein relative to body requirements for specific amino acids, and resulting changes in the arterial pool. As regards amino acid catabolism, the role of the PDV as a site of essential amino acid oxidation, particularly leucine, is emerging. That said, the relationship between net liver uptake of most amino acids and their net PDV release into the portal vein, and resulting net total splanchnic supply to the peripheral tissues, strongly supports the concept of the liver as an integrator of absorptive supply and requirement, but apart from variations in blood concentration the mechanisms controlling this integration are poorly understood. The exception in this regard are the branched chain amino acids, which are not subject to extensive liver catabolism, especially when supply is more limiting relative to demand, and are catabolized in the PDV and other extra-hepatic body tissues. The costs of feeding excess protein include increased heat production, which occurs both in the PDV and liver, and reduced net energy supply. This increase in heat energy is not due to increased ureagenesis from ammonia per se, and excessive ammonia absorption does not appear to increase liver catabolism of amino acids as has been hypothesized. As in nonruminants, splanchnic metabolism of amino acids in ruminants, and the efficiency of their post-absorptive use, varies with supply and demand. When amino acids are absorbed in excess or requirement, the excess supply is catabolized, with a part of that catabolism occurring within the splanchnic tissues. However, both amino acid supply and requirement are determined by the amount and form of dietary energy supply.

Keywords: portal-drained viscera, liver, amino acids, ammonia, urea

Introduction

Historically, most protein rationing systems for ruminants have considered the productive utilization of dietary nitrogen (N) or amino acids (AA) as a fixed proportion of the amount of protein digested, or the amounts of individual amino acids predicted to be absorbed from the small intestine, which is often called 'metabolizable protein' (AFRC, 1998; NRC, 2001; MacRae *et al*., 1988). However, as in nonruminants, productive response (i.e. milk protein yield) to increased amino acid supply is typically nonlinear or biphasic (Whitelaw *et al*., 1988; Doepel *et al*., 2004; Aikman *et al*., 2002). Indeed, in abomasal or duodenal infusion studies, the milk protein response to increased postruminal supply of amino acids or protein varies hugely, in part due to variation in the basal dietary and microbial protein supply relative to requirements or the potential milk protein response (Aikman *et al*., 2002; Doepel *et al*., 2004; MacRae *et al*., 1988) and nutrient partitioning between milk and body tissues (Whitelaw *et al*., 1988). To improve our ability to predict the productive use of dietary N, and feed ruminants more efficiently and with less environmental N loss, it has long been purported that the fractional use of individual AA, which is in part determined by their metabolism within specific tissues of the body, must be better defined (AFRC, 1998; MacRae *et al*., 1988). Conceptually, empirical representations of N utilization within the cow have been compared to 'black box' approaches, whilst those seeking to mathematically represent amino acid utilization in more mechanistic terms have stressed the need for shining a light into the 'black box' which is amino acid metabolism in the animal, with the goal of defining the metabolic 'road maps' of nutrient utilization, and predicting the productive use of amino acids and other nutrients for milk or meat production. To date, however, these efforts have typically resulted in the mathematical representation of the cow as an even greater number of 'black boxes' representing specific tissue beds, which are empirically linked, or perhaps a slightly greyer box rather than a black one.

In order to further this process of mapping nutrient and metabolite flows, a considerable effort has been given to quantifying the metabolism of specific nutrients by the splanchnic tissues and/or mammary gland under varying conditions of nutrient supply and demand (for reviews see e.g. Lapierre and Lobley, 2001; Reynolds, 2002). Whilst many dictionaries define the word 'splanchnic' as referring to the viscera generally, physiologists have historically restricted the term 'total splanchnic tissues' to the tissues of the portal-drained viscera (PDV) and liver combined. The PDV tissues are critical to the amino acid economy of the animal, as they include the gastrointestinal tract (GIT), as well as the pancreas, spleen and a substantial amount of associated adipose tissue drained by the portal vein. Amounts of microbial, feed and endogenous amino acids reaching the small intestine and absorbed into the mesenteric veins are determined by events occurring in the lumen of the GIT and small intestinal enterocytes, where amino acid absorption occurs. An integral component of this digestive process is the cycling of endogenous N from blood to the lumen of the GIT via urea and endogenous amino acids and through ammonia and amino acid absorption into blood (Lapierre and Lobley, 2001). All portal blood passes through the liver before reaching other tissues, and the liver removes most of the ammonia absorbed into the portal vein and converts it primarily to urea and other non-toxic compounds. In addition, liver metabolism can have an immediate impact on the availability of amino acids and other nutrients during their absorption into the body, or what is often referred to as their 'first-pass' through the liver. Indeed, through numerous neural, peptide, and other yet to be determined

signaling pathways there is an elegant integration of nutrient supply from the PDV and body demands which is orchestrated by liver metabolism.

In addition to their roles in diet digestion and nutrient absorption and assimilation, the splanchnic tissues also have a number of important 'service' functions, including the catabolism of 'surplus' amino acids, the detoxification of ammonia, and numerous immune functions. As a consequence of these critical support functions the splanchnic tissues have a disproportionately high rate of metabolism and a particularly high rate of protein synthesis relative to other body tissues. In addition, the liver has an important role in maintaining the body's glucose balance, which represents an important component of amino acid carbon utilization. Therefore, the splanchnic tissues are important to the amino acid economy of the ruminant not only in terms of amino acid supply, but also in terms of the extent to which they contribute to the amino acid turnover and oxidative or endogenous loss (i.e. maintenance requirement). The present paper will consider the measurement of splanchnic amino acid metabolism and the impact of the splanchnic tissues on amino acid supply, as well as the potential metabolic implications of nonprotein N cycling.

Measurement of splanchnic amino absorption and metabolism

To obtain in vivo measurements of amino acid absorption and metabolism by splanchnic tissues in ruminants, there are 3 basic techniques that have been used, alone or in combination. First, appropriately placed gastrointestinal cannulas can be used to measure the net flow of amino acids into and out of the small intestine, or their net disappearance from the lumen of the gut, which underestimates true rates of absorption to the extent that endogenous amino acids contribute to amino acid flow at the ileum (Fuller and Reeds, 1998). The technique has been used extensively in pigs (Fan and Sauer, 2002), and data are available for nonlactating ruminants, but published measurements of small intestinal disappearance of amino acids in lactating dairy cows are scarce (Sutton and Reynolds, 2003). This reflects the difficulty of establishing and maintaining ileal cannulas, as there is vastly more data available in the literature describing duodenal flow of amino acids in lactating dairy cows (NRC, 2001).

A second approach which has been used to obtain measurements of amino acid absorption and metabolism by splanchnic tissues is the use multiple chronic blood sampling catheters, strategically placed to measure venous-arterial concentration difference (VA) for amino acids and blood or plasma flow rate across the PDV or anterior mesenteric-drained viscera (MDV; Lindsay and Reynolds, 2005). Net flux of amino acids can then be calculated as the mathematical product of VA and flow, with positive rates denoting net release or absorption of amino acids into venous drainage, and negative rates denoting net removal from arterial blood by the tissues contributing venous blood at the point of sampling. As for measurements of net disappearance from the lumen of the gut, these net measurements underestimate the true rate of absorption, in this case to the extent that individual amino acids are removed from arterial blood or sequestered by small intestinal enterocytes during absorption from the gut lumen.

Thirdly, isotopic labeling can be used in combination with gut cannulation or blood vessel catheterization to quantify the metabolism of individual amino acids in more detail. For example, combining isotopic labeling of blood amino acid or N pools with measurements of the

appearance those components in gut contents using cannulation techniques can provide estimates of endogenous secretions appearing in the duodenum or ileum (Lien *et al*., 1997; Ouellet *et al*., 2002). On the other hand, combining measurements of net amino acid flux across the PDV with isotopic labeling of blood amino acids can provide a measure of the fractional uptake of amino acids from arterial blood by the tissue of the PDV, and by adding the amount extracted to net flux the unidirectional rate of release into venous blood can be calculated. Finally, by introducing a label into the lumen of the small intestine, utilization of amino acids during their absorption by the small intestinal enterocytes can be ascertained (MacRae *et al*., 1997a; Lindsay and Reynolds, 2005). However, this later approach requires that additional label be introduced into blood to correct for the extraction of label absorbed from the gut lumen which is subsequently extracted from arterial blood by the PDV. Failure to account for this extraction of label which is re-circulated to the PDV in arterial blood can result in excessive estimates of 'first-pass' metabolism.

Metabolism of amino acids by the portal-drained viscera

As a consequence of their many critical functions for maintenance and production, the splanchnic tissues have a greater metabolic activity per unit mass than most other body tissues. This is reflected by their rate of blood flow and oxygen consumption, which account for as much as 40 % of cardiac output (Huntington *et al*., 1990) and 50 % or more of whole body oxygen consumption (Reynolds, 1995). This high rate of oxygen consumption is associated with a high rate of protein turnover (synthesis and degradation; Lapierre and Lobley, 2001), and the oxidation of substrates such as acetate, 3-OH-butyrate, glucose, and amino acids (Windmuller and Spaeth, 1980). Therefore, considerable amounts of amino acids are required by the PDV and liver both as precursors for protein synthesis, as well as substrates for oxidative metabolism. Amounts of oxygen consumed by the PDV and liver, and their heat production, are dictated primarily by feed dry matter (DM) and thus metabolizable energy (ME) intake (Lindsay and Reynolds, 2005). In studies conducted at USDA, Beltsville, MD and The University of Reading, England, we have obtained about 500 daily measurements (each an average of 5 to 12 hourly or half-hourly measurements) of splanchnic metabolism in beef and dairy cattle across a range of dietary intakes and productive states (Reynolds, 2005), including 265 measurements of splanchnic oxygen consumption (Reynolds, 2002). In these data the relationship between DM intake and splanchnic oxygen consumption is apparent, although the variation observed, especially in lactating dairy cows, emphasizes the importance of other factors, such as gut fill and mass and the muscular work of digestion (Reynolds *et al*., 1991a) and the metabolic activity of the liver required to support milk synthesis in early lactation (Reynolds *et al*., 2003) as determinants of total splanchnic oxidative metabolism. Whilst these tissues have a high rate of oxidative metabolism, they also have access to a large portion of absorbed nutrients, both during the absorption processes, but also from the disproportionate amount of total arterial supply (i.e. cardiac output) they receive.

Portal-drained visceral metabolism of amino acids during their absorption versus arterial supply

Measurements obtained using isolated loops of the rat small intestine indicated the small intestinal mucosa preferentially uses luminal aspartate and glutamate and arterial glutamine, as well as 3-OH-butyrate and glucose, to meet their oxidizable carbon requirements (Windmueller and

Spaeth, 1980). The substantial impact of small intestinal metabolism on glutamate, glutamine, and glucose supply has also been documented for the neonatal pig (Stoll *et al*., 1999). Because of this high rate of metabolism and the requirement of the small intestine for specific nonessential amino acids (NEAA), a logical assumption has been that substantial amounts of other amino acids are subjected to 'first-pass' metabolism during their absorption from the lumen of the gut, and that the metabolism of the small intestine limits the general availability of amino acids to other body tissues. This view was supported by comparisons of the net appearance of amino acids in the portal vein with their net disappearance from the lumen of the small intestine (Reynolds, 2002). In the classic study of Tagari and Bergman (1978), the net appearance of amino acids in the portal vein, measured using multicatheterization techniques, was compared to the net disappearance of those amino acids from the small intestine, measured simultaneously by gut cannulation in 2 sheep. In those sheep, net PDV appearance averaged from less than 0 to 85% of individual amino acid disappearance from the lumen of the small intestine, with particularly low recoveries or net losses (net use from arterial blood) for aspartate/asparagine and glutamate/glutamine (asparagine and glutamine are converted to aspartate and glutamate, respectively, during acid hydrolysis of digesta). The highest recovery was observed for alanine, which is both absorbed from the diet and also synthesized and released by the PDV (and other tissues) as a means of transferring N from catabolized amino acids to the liver for ureagenesis. Similar observations have been reported for neonatal pigs (Stoll *et al*., 1998; Bos *et al*., 2003), with the recovery of dietary EAA in milk replacer as increased net PDV absorption ranging from 32 to 78%, and the recovery of alanine, arginine and tyrosine equal to or exceeding intake (96 to 205%). In the case of tyrosine the higher recovery was attributed to hydroxylation of phenylalanine, whilst arginine synthesis from citrulline may have accounted for the additional arginine released into the portal vein (Stoll *et al*., 1998). These observations were comparable to previous observations in older pigs (e.g. Rerat *et al*., 1988).

Similar comparisons in ruminants are rare (MacRae *et al*., 1997b; Berthiaume *et al*., 2001), as amounts of amino acids absorbed from the small intestine cannot be estimated from intake, but must be directly measured using duodenal and ileal cannulas. In a study in 3 dairy cows fed a total mixed ration (TMR) at 2 DM intakes in late lactation and the subsequent dry period (Reynolds *et al*., 2001a; Table 1), comparison of net disappearance of amino acids from the small intestine with their net PDV appearance also show a considerable discrepancy, suggesting a substantial utilization of amino acids in the PDV which limits the net supply of absorbed amino acids to other body tissues. The 'portal recovery' of amino acids disappearing from the small intestine varied considerably, and thus there were few significant effects of level of DM intakes, physiological stage (dry versus lactating), or their interaction on the net recovery of amino acids in the portal vein. Numerically, the percentage recovery of most essential amino acids was lower at higher DM intake during late lactation, but greater at higher DM intake during the dry period. As in nonruminants, the net recovery of alanine, arginine and tyrosine were high relative to the other amino acids measured, as were recoveries of methionine and serine, whilst the net recoveries of many of the nonessential amino acids, in particular the glutamate/glutamine and aspartate/asparagines pairs, were low. However, the extent to which this discrepancy arises as a consequence of 'first-pass' metabolism is impossible to ascertain solely from measurements of net PDV flux.

Table 1. Dry matter intake (DMI), milk yield, and net PDV recovery (%) of amino acids absorbed from the small intestine (duodenal minus ileal flow plus endogenous ileal flow) in 3 dairy cows fed at 2 DMI of a lactation TMR during the dry period and during lactation. Data from Reynolds et al. *(2001a).*

	Dry period		Lactation	
	Low	High	Low	High
DMI, kg/d	8.0	12.0	14.9	19.4
Milk yield, kg/d	–	–	14.3	16.5
Net PDV flux/SI disappearance, %				
Valine	19	39	46	29
Leucine	27	39	44	34
Isoleucine	28	40	46	38
Methionine	61	76	70	56
Lysine	43	60	55	45
Threonine	29	47	43	38
Phenylalanine	48	61	57	45
Histidine	24	51	54	42
Total essential	33	49	50	39
Arginine	28	50	61	49
Alanine	57	86	60	61
Glycine	37	42	29	37
Serine	59	80	65	58
Aspartate	22	29	23	20
Glutamate	-21	-2	12	12
Proline	34	31	33	24
Tyrosine	60	72	73	58
Total nonessential	33	46	40	38
Total	33	47	45	38

When interpreting measurements of net PDV amino acid metabolism obtained in vivo using multicatheterization techniques, it is critical to remember that the PDV is a heterogonous collection of tissues which are simultaneously releasing amino acids into venous blood, removing them from arterial blood, and in some cases releasing them into the lumen of the gut as well. Thus amino acid absorption from the lumen of the gut and transfer to blood by small intestinal enterocytes, which represent a relatively small proportion of the total mass of the PDV, is counterbalanced by amino acid uptake from arterial blood by other PDV tissues, such as the stomach and hindgut, and the enterocytes themselves (van Goudoever *et al.*, 2000; Bos *et al.*, 2003). This is clearly illustrated by the fact that net amino acid release by the PDV is lower than net release by the MDV, which does not include the stomach tissues and upper small intestine, where little amino acid absorption occurs. In sheep, net PDV release of essential amino acids (EAA) averages about 2/3 (MacRae *et al.*, 1997b) to 3/4 (Remond *et al.*, 2003) of net MDV release, suggesting that amino acid utilization by the rumen and other stomach tissues account for roughly 1/3 to 1/4 of the amino acids absorbed on a net basis into the mesenteric veins. The extent to which measurements of net MDV flux include metabolism of amino acids from the arterial blood by

the hindgut will depend on the location of the anterior mesenteric vein sampling catheter, but in sheep net MDV flux should exclude hind gut metabolism (MacRae *et al.*, 1997a; Remond *et al.*, 2003). Regardless, comparisons of net MDV and PDV amino acid flux clearly illustrate that the uptake of amino acids from arterial blood by tissues other than the absorptive cells of the small intestine account for a portion of the absorbed amino acids that do not appear in the portal vein on a net basis.

Sources and fates of amino acids sequestered by the portal-drained viscera

As discussed previously, measurements of absorptive or 'first-pass' sequestration of absorbed amino acids can be obtained in ruminants using isotopic labeling of luminal amino acids, coupled with measurements of the extraction of absorbed label re-circulated to the PDV in arterial blood (MacRae *et al.*, 1997a; Yu *et al.*, 2000). Similar models of first-pass and arterial or 'systemic' amino acid use by the PDV have also been developed and used in studies of PDV amino acid metabolism in pigs (e.g. van Goudoever *et al.*, 2000; Bos *et al.*, 2003). Using this approach in sheep fed dried lucerne pellets at 2 intakes, MacRae *et al.* (1997a) found that for the branched chain amino acids, lysine, threonine, and phenylalanine, total PDV sequestration equaled substantial portions of whole body irreversible loss (from 42 to 67%). Of this total PDV sequestration, the majority was derived from arterial blood (75 to 87 %), as opposed to 'first-pass' or absorptive use during absorption from the lumen of the gut. Histidine metabolism was also measured, but PDV histidine metabolism differed from the other EAA by the fact that 50% of total PDV sequestration arose from absorptive metabolism. This was not due to greater absorptive use of histidine, but the fact that the amount sequestered from arterial blood was lower relative to the other EAA. In total, PDV sequestration of histidine accounted for only 32% of total irreversible loss in the body, the lowest proportion for the seven EAA measured.

Amino acids sequestered by the PDV can be used for synthesis of constitutive or secreted protein, or oxidized. As discussed previously, specific nonessential amino acids have been identified as preferred oxidative substrates for the intestinal enterocytes. In a study in the neonatal pig (Stoll *et al.*, 1999), luminal (absorptive use) glutamate (36%), arterial glutamine (15%) and arterial (29%) and luminal (6%) glucose could account for 87% of total CO_2 production by the PDV. For the EAA sequestered in the PDV, an obvious fate is the replacement of endogenous proteins secreted or sloughed into the lumen of the gut. Endogenous amino acids entering the rumen and small intestine lumen would be available for re-absorption, or their N could be re-absorbed as ammonia or amino acids synthesized by microbial protein. The supply of endogenous amino acids to the duodenum in part explains the discrepancy between intestinal disappearance and portal appearance of amino acids in ruminants (Table 1). Although the extent of amino acid absorption from the hindgut in mature animals needs to be addressed, particularly for certain amino acids for which there is evidence they may be absorbed from the large intestine of the pig (Fuller and Reeds, 1998), endogenous amino acids entering the hindgut would largely be unavailable for re-absorption and they or their N would be excreted in feces. Studies in the small intestine of pigs suggest that 65% of the amino acids in intestinal secretions and cells sloughed into the lumen of the small intestine are re-absorbed or 're-cycled' (Bos *et al.*, 2003). Similar estimates are not available for ruminants, but based on estimates derived from labeling of N pools

using 15N, Ouellet *et al.* (2002) estimated that endogenous N secretions represented 30% of total protein synthesis in the digestive tract.

The differential labeling model developed by MacRae *et al.* (1997a) was used in sheep to ascertain the extent to which leucine sequestered during arterial or absorptive metabolism was oxidized or used for protein synthesis by the PDV and MDV (Yu *et al.*, 2000). Of the total leucine sequestered by the PDV, 86 % was derived from arterial blood, and only 12% of that arterial use occurred in the MDV. Of the leucine sequestered, 16% was oxidized, and about half (44%) of PDV leucine oxidation occurred in tissues drained by the anterior mesenteric vein (mostly the small intestine). This was largely a consequence of the oxidation of leucine derived from arterial blood, as only a fraction (1%) of the leucine sequestered during absorption was oxidized. This suggests that virtually all the leucine sequestered during absorption was used for the synthesis of constitutive and secreted or sloughed proteins in the enterocytes of the intestine. On the other hand, the data also suggest that a substantial amount of the leucine sequestered from arterial blood is used for anabolic purposes in tissues other than the MDV (the stomach, hindgut, spleen and pancreas). Other studies in sheep found that the MDV accounted for 40% of the PDV oxidation of leucine, and that PDV oxidation accounted for 25% of whole body leucine oxidation (Lobley *et al.*, 2003). Oxidation of methionine across the PDV was also measured, with 60% occurring in the MDV, but accounting for only 10% of whole body oxidation. In contrast, the oxidation of phenylalanine and lysine was not measurable across the PDV under the conditions employed (Lobley *et al.*, 2003). These data suggest that in addition to certain NEAA, considerable catabolism of leucine occurs in the PDV of ruminants. In nonruminants, leucine and the other branched chain amino acids are unique in that the majority of their catabolism occurs in muscle and other tissues other in than the liver (Layman, 2003). The data from sheep suggest that a substantial portion of this catabolism occurs in the tissues of the PDV, with a disproportionate amount (40 % or more) of oxidation occurring in the MDV. While the mass of the MDV would account for considerably less than 40% of the total PDV tissues, the MDV accounts for roughly 40 % of total PDV blood flow (Yu *et al.*, 2000). In lactating dairy cows, the small intestine accounted for only 10% of the total mass of the PDV, and only 20% when mesenteric and omental fat are excluded from the total (Gibb *et al.*, 1992).

Considerably more data describing PDV utilization of dietary amino acids during their absorption ('first-pass') and from arterial (systemic) blood are available for pigs, to a large extent as a consequence of the elegant studies using the neonatal pig models developed by P. J. Reeds, D. G. Burrin and their colleagues at the USDA/ARS Children's Nutrition Research Center in Houston. In considering those data relative to amino acid metabolism in the PDV of ruminants, it is important to remain cognizant of a number of species differences, including the extensive development of the ruminant stomach and the substantial postprandial variations in digesta flow and nutrient absorption which occur in nonruminants. For example, Bos *et al.* (2003) demonstrated substantial repartitioning of PDV lysine sequestration between absorptive and arterial supplies as animals progressed through an absorptive 'cycle' consisting of the period before feeding and 2 subsequent 4 h periods immediately after feeding. In the absence of dietary amino acids before feeding, the PDV used systemic lysine exclusively, whilst immediately after feeding first-pass metabolism was the predominant source of lysine used by the PDV. During the later half of the absorptive period, less first-pass metabolism occurred, but there was an increase

in the absorption of 're-cycled' lysine into the portal vein. This re-cycled lysine was derived from endogenous sources which contained labeled lysine sequestered during the early absorptive period. Similar variation in the source of amino acids metabolized by the PDV may occur in ruminants as well, but the more constant flow of digesta and patterns of amino acid absorption in ruminants likely precludes such dramatic shifts in amino acid metabolism between luminal and arterial supplies.

Research using stable isotopes to trace amino acid metabolism in piglets has also demonstrated effects of dietary protein supply on the extent of absorptive and arterial amino acid utilization (van Goudoever *et al*., 2000). In piglets fed a high (normal) protein diet, lysine utilized by the PDV was derived entirely from arterial supply, with no measurable absorptive use. When a low protein diet (40 % of normal) was fed, first-pass absorptive utilization increased markedly, accounting for 42 % of total PDV use of lysine, which in total remained unchanged. These shifts in lysine use from arterial supply to absorptive use of dietary lysine were determined, or at least associated with, changes in arterial concentration of lysine (van Goudoever *et al*., 2000; Bos *et al*., 2003). In both studies, fractional uptake of arterial lysine by the PDV, and blood flow, remained constant, thus mathematically the total amount extracted was determined by arterial concentration. Similarly, in lactating dairy cows abomasally infused with increasing amounts of casein, net PDV utilization of amino acids was linearly related to supply, with the majority of that supply derived from arterial blood (Hanigan *et al*., 2004b). These observations suggest that arterial supply is an important determinant of the total utilization of lysine, and other amino acids, by the PDV.

Arterial concentrations of amino acids will largely be determined by amounts absorbed from the diet relative to body requirements (Reynolds, 2002), thus for some amino acids (eg the branched chains and lysine), the PDV may contribute to catabolic regulation of amino acid balance. This concept is supported by the fact that in pigs fed a high protein diet the PDV accounted for 31 % of body lysine oxidation, but there was no measurable oxidation of lysine by the PDV when a low protein diet was fed (Van Goudoever *et al*., 2000). In this regard, the lack of measurable lysine oxidation by the PDV of sheep (Lobley *et al*., 2003) may reflect the lysine status of the sheep used. Other studies have shown that changing pigs from a high- to a low-protein diet did not change total CO_2 production by the splanchnic tissues, but the amount of leucine and glutamate oxidation by the PDV was reduced for the low protein diet, whilst the oxidation of glucose increased in compensation (van der Schoor *et al*., 2001). In lactating and dry dairy cows (Reynolds *et al*., 2001a), absorptive use of leucine and phenylalanine was minimal at lower DM intake of a lactation TMR, but both absorptive and arterial sequestration increased with greater intake within both physiological stages. For both amino acids, PDV sequestration accounted for a greater fraction of total absorption in dry cows and at higher intakes within each physiological state, suggesting a greater utilization by the PDV with greater supply relative to requirement. Similarly, the amount of absorptive and arterial EAA use increased with greater DM intake in sheep (MacRae *et al*., 1997a). In dairy cattle (Reynolds *et al*., 2001a) and sheep (MacRae *et al*., 1997a), absorptive use accounted for a greater portion of total PDV use of phenylalanine than observed for leucine. This was due to greater sequestration of arterial leucine, which may reflect differences in the amino acid concentration of constitutive proteins of the PDV (MacRae *et al*., 1997a).

Liver metabolism of amino acids

Nutrients absorbed into the portal vein must traverse the liver before reaching the vena cava and ultimately the rest of the body. Like the PDV, the liver is extremely metabolically active, accounting for 2 to 3 % of body mass, but as much as 25 % of body oxygen consumption (Reynolds, 1995). In addition to the blood supplied by the portal vein, the liver also receives oxygen, and nutrients, from the hepatic artery, which contributes 10 to 20 % of liver blood flow in ruminants. The high rate of liver metabolism is a consequence of numerous critical functions, many of which involve amino acid metabolism. These functions include the synthesis of urea, glucose and constitutive and transport proteins, the catabolism of amino acids whose availability exceeds requirement, and the transamination of amino acids that are participants in interorgan carbon and nitrogen shuttles which involve the liver. The liver is often described as the 'integrator' of dietary supply with body requirements, and there is no question that the liver is an important site of amino acid metabolism where substantial amounts of amino acids are removed relative to their absorption into the portal vein (Elwyn, 1970). The extensive catabolism of amino acids by the liver, and its anatomical position, suggests that the liver dictates amino acid supply to peripheral tissues, but the liver also responds to changes in arterial concentration of amino acids, which is determined by both their rate of absorption and the extent of their utilization by other body tissues.

Liver amino acid removal relative to absorptive versus arterial supply

On a net basis, liver removal of a number of amino acids is high relative to amounts absorbed across the PDV (for review see Lobley *et al*., 2000). In maintenance fed steers (Reynolds *et al*., 1994), late gestation dairy cows (Wray-Cahen *et al*., 1997), or maintenance fed sheep (Lobley *et al*., 2001), the liver typically removes substantial quantities of individual amino acids on a net basis relative to their net absorption into the portal vein. This is particularly true for the nonessential amino acid alanine, glycine and serine, and in many cases glutamine, where net liver removal may be equal to or in some cases exceed net PDV release (see Lobley *et al*., 2000). A negative net total splanchnic release of an individual amino acid (i.e. greater net liver removal than net PDV release), such as alanine or glycine, reflects a net contribution of nonsplanchnic tissues (e.g. skeletal muscle) to liver removal of these amino acids, which would be expected under certain nutritional conditions considering the role of these amino acids in the interorgan transfer of carbon and nitrogen between the liver and other tissues (Lobley *et al*., 2000). Alanine, glycine, and other amino acids synthesized via transamination in muscle and other tissues transport N from the catabolism of other amino acids to the liver for ureagenesis (Bergman and Heitmann, 1980). It should also be remembered that net flux of an amino acid across the liver represents the sum of unidirectional removal and release, thus for many amino acids which can be released by the liver their unidirectional rate of removal may be greater than indicated by net flux measurements (e.g. glutamine; Bergman and Heitmann, 1980).

While net liver removal of amino acids may equal a substantial portion of net PDV release, the total supply of amino acids to the liver in blood is much greater because the majority of the amino acids in the portal vein are from the arterial circulation. As the liver receives as much as 40% of cardiac output, it also receives a similar proportion or the arterial pool of amino acids. Depending on the amino acid, the net addition of absorbed amino acids into the portal vein is typically

small relative to the total supplied to the PDV in arterial blood. Across 86 observations (each representing an average of from 6 to 8 hourly measurements) from 8 experiments conducted at the University of Reading (Reynolds, 2005), net PDV release of individual amino acids averaged 16% of their supply in arterial blood received by the PDV (Table 2). Therefore, net liver removal is always lower when expressed as a fraction of total supply (Table 2) rather than as a fraction of net PDV release (Table 3). Mathematically, this means that for each pass of cardiac output through the liver the amino acids removed have a greater chance of being from the arterial blood delivered

Table 2. Total arterial blood supply of amino acids (AA) to the PDV (PDV input), net PDV release of those AA as a percentage of total arterial input, supply of AA to the liver in hepatic arterial blood, and net liver removal as a percentage of total portal vein and hepatic arterial blood supply. Means of 86 observations of splanchnic amino acid flux (6 to 8 hourly measurements each) from lactating and dry dairy cattle in 8 experiments. Measurements obtaining during infusions of AA or protein excluded.

	mmol/h		% of input		mmol/h		% of total input	
Amino acid	**PDV input**	**Std Dev[1]**	**PDV release**	**Std Dev**	**Arterial input**	**Std Dev**	**Liver removal[2]**	**Std Dev**
Valine	351.9	153.3	8.3	6.0	84.9	52.3	-0.6	4.1
Leucine	196.5	79.2	19.5	9.9	47.8	28.3	-1.1	4.3
Isoleucine	169.3	62.5	15.1	7.5	41.0	23.3	-1.8	5.5
Methionine	24.4	6.6	43.5	16.2	6.1	3.2	9.9	8.0
Lysine	115.6	36.6	30.1	12.2	27.9	14.8	2.2	6.9
Threonine	143.6	43.9	17.5	6.8	35.8	19.4	5.3	4.8
Phenylalanine	60.8	16.7	35.9	9.0	15.0	7.8	12.4	5.5
Tryptophan	47.5	16.2	8.7	10.5	11.6	6.3	4.2	5.2
Histidine	58.0	27.0	17.0	10.3	14.3	9.4	4.8	7.2
Total essential	1194.6	374.7	16.4	6.4	291.6	155.1	1.7	4.3
Arginine	105.4	33.0	19.7	9.0	25.6	13.4	4.9	7.4
Citrulline	103.7	30.7	8.3	4.6	26.3	16.2	0.8	4.6
Ornithine	75.7	28.9	14.0	8.1	18.7	11.9	-3.2	4.5
Alanine	273.6	88.0	22.9	7.7	67.0	34.7	11.3	5.3
Glycine	304.1	141.6	14.8	7.9	77.0	52.4	14.9	6.6
Serine	114.9	38.1	31.2	10.8	28.6	16.1	19.4	6.2
Aspartate	20.1	11.8	18.4	13.4	4.8	3.6	2.4	12.7
Asparagine	36.9	16.6	56.4	32.7	9.2	5.7	16.7	14.2
Glutamate	94.8	33.3	6.5	8.2	23.4	14.6	-23.7	15.5
Glutamine	249.5	72.4	5.2	8.2	62.3	34.3	5.7	6.4
Proline	101.3	34.0	15.7	6.9	25.3	14.7	6.5	7.9
Tyrosine	64.1	21.4	29.8	7.9	15.7	8.2	11.0	5.3
Total nonessential	1520.7	378.9	15.8	6.5	374.6	195.4	7.7	4.9
Total	2716.0	710.8	15.8	5.6	663.3	343.9	5.0	4.4

[1]Standard deviation.

[2]For liver removal, negative rates denote net liver release.

Table 3. Net liver removal of amino acids (AA) as a percent of their net PDV release. Data from 8 growing beef steers fed 75% concentrate diets containing 12 or 16% crude protein (CP) at 2 metabolizable energy (ME) intakes (Reynolds et al.*, 1992 and 1995) and from 3 dairy cows fed 2 dry matter (DM) intakes of a lactation TMR during late lactation and the subsequent dry period (Reynolds* et al.*, 2001a).*

	Beef steers				Dairy cows			
	12 % CP		16% CP		Dry period		Lactating	
Intake	**690 MJ**	**904 MJ**	**690 MJ**	**904 MJ**	**Low**	**High**	**Low**	**High**
DM intake, kg/d	5.1	6.9	5.0	7.0	8.0	12.0	14.9	19.4
N Intake, g/d	96	128	131	179	235	345	416	523
Net PDV release, mmol/h								
Essential AA	44.4	72.6	44.6	89.4	42.0	78.6	151.7	152.2
Nonessential AA	38.2	71.5	42.0	96.2	62.3	109.3	180.0	213.9
Total AA	82.6	144.1	86.7	185.5	104.4	187.8	333.2	364.6
Liver removal, % of net PDV release								
Methionine	40	52	67	92	96	121	49	51
Lysine	28	23	19	63	46	63	4	9
Phenylalanine	87	76	91	87	146	122	65	69
Branched chains	10	-16	2	41	28	-6	-13	-31
Essential AA	37	16	40	47	71	54	14	9
Alanine	144	78	68	77	106	114	82	90
Nonessential AA	74	51	62	83	147	125	68	67
Total AA	54	33	51	66	116	96	43	43

to the PDV, rather than an amino acid newly arrived in the portal vein from the lumen of the small intestine. However, it should be noted that for the essential amino acids methionine, lysine, and phenylalanine, net PDV absorption represents a greater fraction of total arterial supply to the PDV (Table 2) than observed for most other amino acids. For methionine, this reflects a lower arterial concentration and systemic supply to the PDV.

When mixtures of exogenous amino acids have been incrementally infused into the mesenteric vein, to mimic increased absorption, the fractional clearance of individual amino acids by the liver has in some cases remained constant (Lobley *et al.*, 2001; Wray-Cahen *et al.*, 1997), suggesting that liver removal of amino acids increases linearly in response to increased input, and that fractional clearance remains unchanged. However, these responses were measured during short-term infusions, without time for adaptation, and in animals fed near maintenance. As noted by Lobley *et al.* (2000) and Blouin *et al.* (2002), fractional removal of amino acids by the liver can vary with changes in dietary inputs or requirements. This is clearly illustrated by data from 2 studies presented in Table 3. In growing beef cattle fed 2 diets differing in crude protein concentration at 2 ME intakes and in dry and lactating dairy cows fed the same TMR at 2 intakes, there were clear differences in the extent to which the amino acids absorbed into the portal vein were removed by the liver on a net basis, reflecting differences in the demand for the amino acids supplied in peripheral tissues. For example, with increased intake and net PDV absorption of amino acids in lactating compared to dry dairy cows, net liver removal as a percentage of net

PDV release was lower during lactation (Table 3), which surely must reflect a greater demand for amino acids by the mammary gland. In contrast, liver removal of amino acids as a percentage of their net PDV release increased in lactating dairy cows given 6-day abomasal infusions of casein compared to control infusions, but in this case there was little effect of casein infusion on milk protein yield, thus the majority of the amino acids supplied by casein infusion appeared to be catabolized (Reynolds *et al*., 1999; Reynolds, 2002).

Increased liver removal of amino acids in cows infused with casein was associated with increased arterial concentrations (Reynolds, 2002), reflecting the imbalance between the rate of increase in supply relative to demand, and rates of liver removal of amino acids may be determined to a large extent by their arterial concentration and total supply to the liver (Hanigan *et al*., 2004a; Waterlow, 1999). However, it is unlikely that the integration of amino acid supply and demand is not subject to some form of neuro-endocrine regulation. Across all the measurements of splanchnic amino acid metabolism obtained in 8 studies in dairy cows at the University of Reading, the relationship between the total supply of amino acids to the liver and their net liver removal, corrected for effects of individual studies, varied considerably (Figure 1). For most amino acids the relationship observed had relatively low correlation coefficients, and this was particularly true for those amino acids whose catabolism occurs extensively in extra-hepatic tissues, such as the branched-chains and lysine (Figure 1). As discussed previously, the branched chain amino acids are removed by the liver to a lesser extent than most other amino acids (Lobley *et al*., 2000), and are catabolized for the most part in extra-hepatic tissues, where they can have important regulatory effects, such as the effects of leucine on protein synthesis (Layman, 2003). In lactating dairy cows, a net release of the branched chain amino acids is often observed (Table 3; Blouin *et al*., 2002), which may in part reflect the transamination of their respective keto-acids released from peripheral tissues, or the catabolism of plasma and blood peptides. On the other hand, the correlation between net liver removal and total blood supply to the liver was higher for phenylalanine, which is subject to extensive metabolism in the liver.

In contrast to the relationship between net liver removal and total blood supply of amino acids, the relationship between net PDV release and liver removal of most amino acids measured was more robust (Figure 2), and appeared to be a determinant of net total splanchnic release of amino acids to nonsplanchnic tissues (Figures 3 and 4). While it is tempting to speculate that these observations demonstrate that the liver is dictating the supply of absorbed amino acids to peripheral tissues, the relationship between net total splanchnic release of individual amino acids and their net liver removal as a percentage of their net PDV release is also due to the simple fact that net total splanchnic release is the mathematical sum of net PDV and liver flux. However, what is clear from these data is that changes in net total splanchnic release of amino acids are not solely due to changes in their net PDV release, but the liver is also a determinant of the net release of amino acids by the splanchnic tissues. This is clearly illustrated by the effects of growth hormone (Bruckental *et al*., 1997) or growth hormone releasing factor to growing steers (Lapierre *et al*., 1992). Increases in body protein retention were associated with decreased liver removal of amino acids and urea production, thus increased total splanchnic supply of amino acids for enhanced protein retention were a consequence of decreased liver removal, not increased absorption. However, the extent to which liver removal of amino acids dictates supply

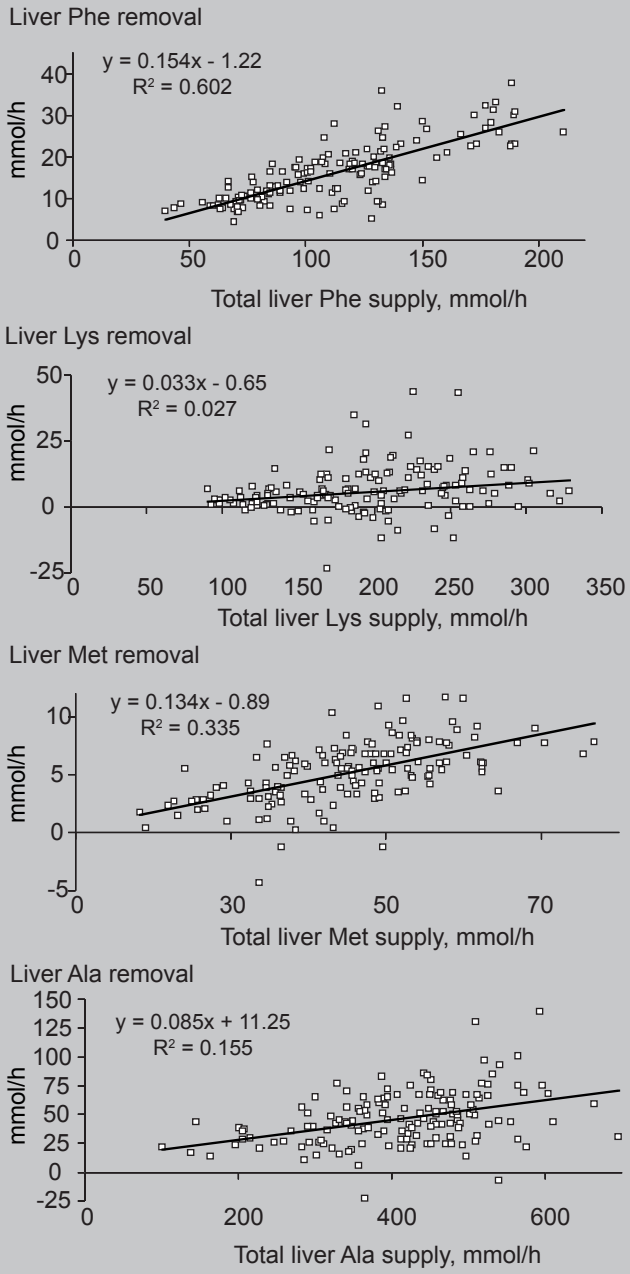

Figure 1. Relationship between net liver removal of selected individual amino acids and their total blood supply to the liver. Data from 8 experiments in lactating (n = 144) and dry (n=6) dairy cows with corrections for effects of experiment.

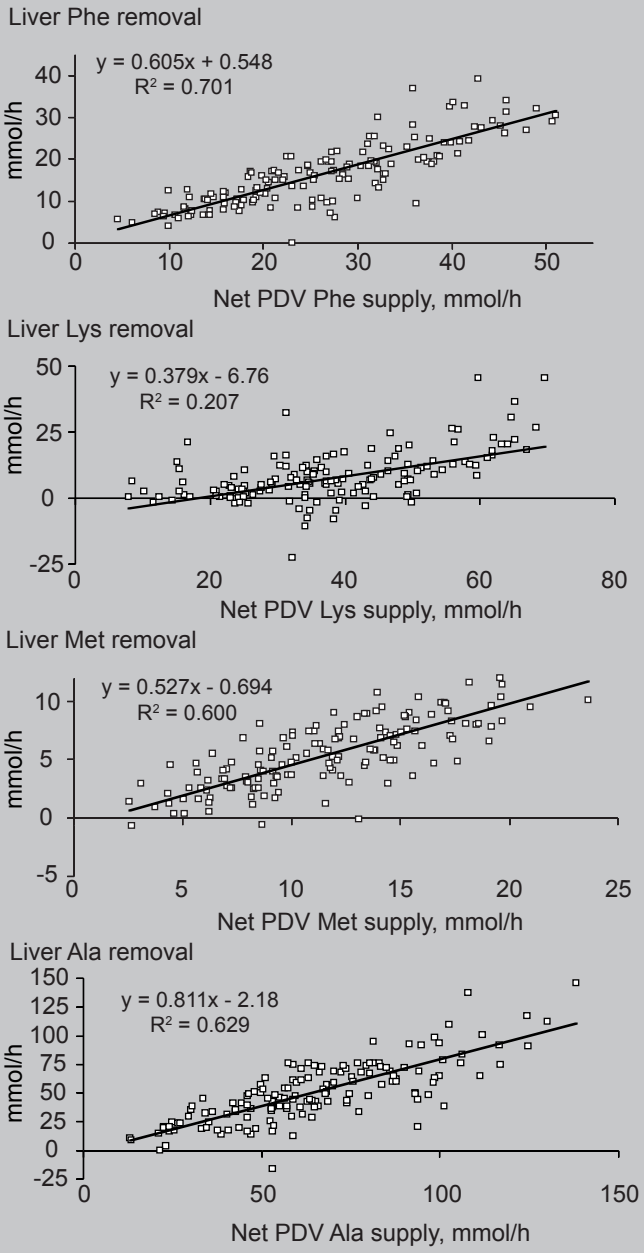

Figure 2. Relationship between net liver removal of selected individual amino acids and their net PDV release. Data from 8 experiments in lactating (n = 144) and dry (n=6) dairy cows with corrections for effects of experiment.

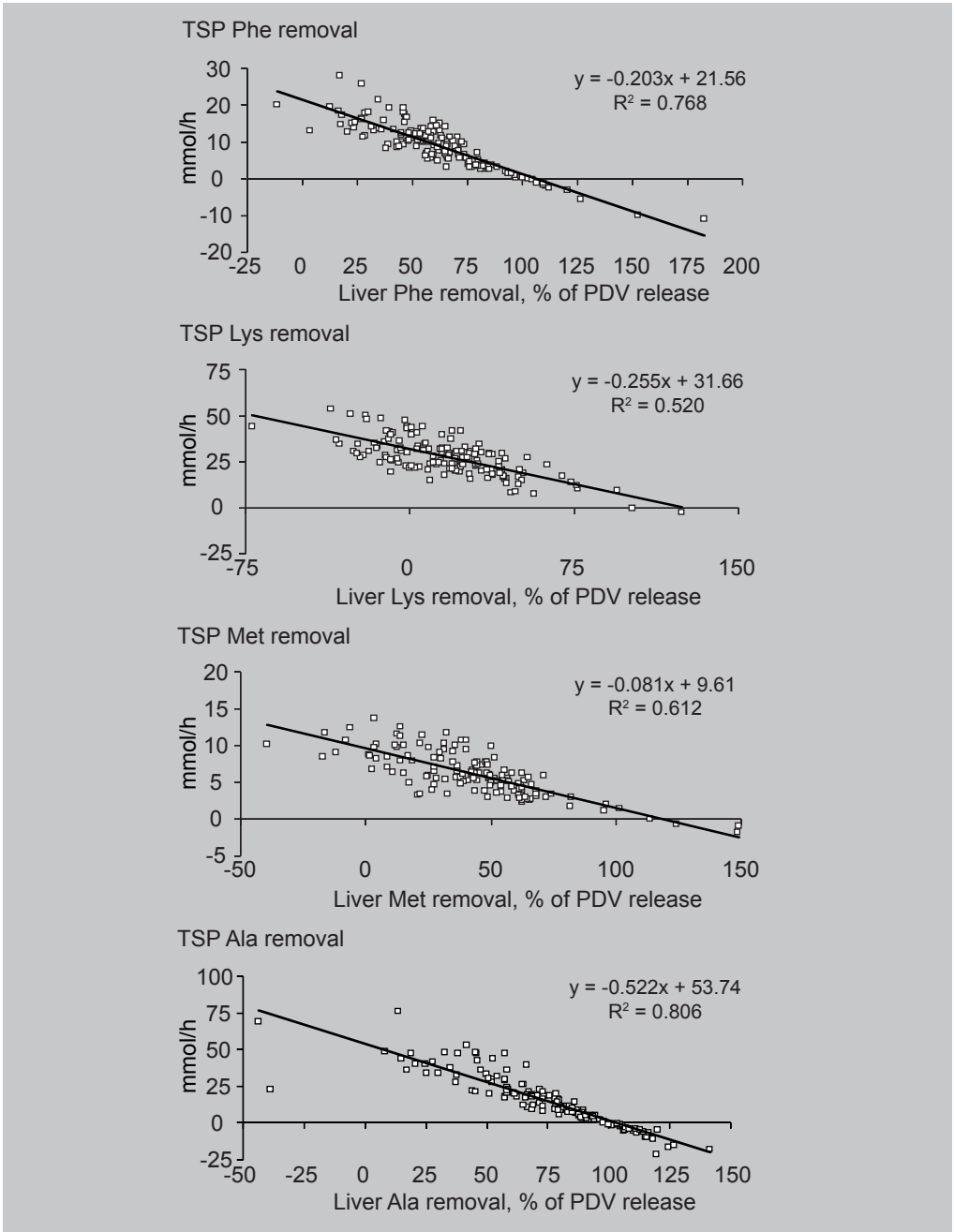

Figure 3. Relationship between net total splanchnic (TSP) release of selected amino acids and their net removal by the liver expressed as a percentage of their net PDV release. Data from 8 experiments in lactating (n = 144) and dry (n=6) dairy cows with corrections for effects of experiment.

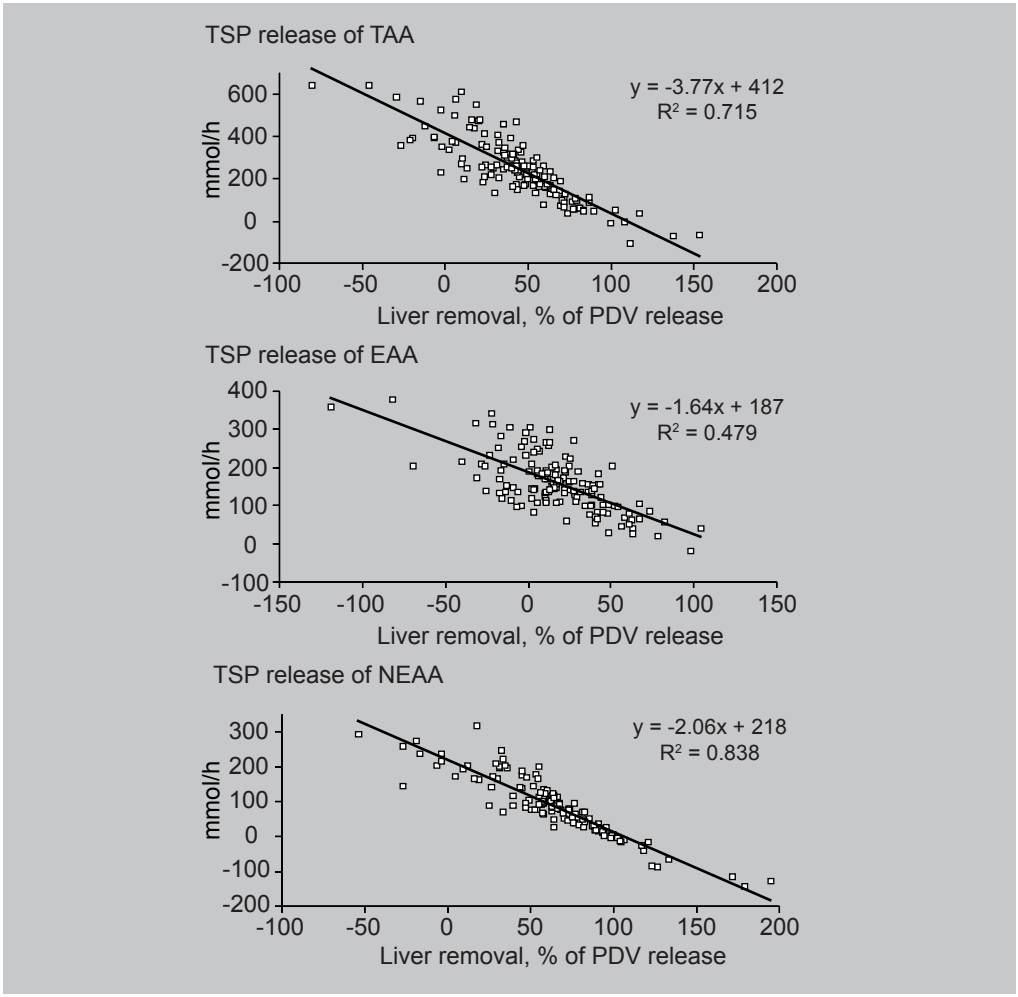

Figure 4. Relationship between net total splanchnic (TSP) release of total (TAA), essential (EAA) and nonessential (NEAA) amino acids and their net removal by the liver expressed as a percentage of their net PDV release. Data from 8 experiments in lactating (n = 144) and dry (n=6) dairy cows with corrections for effects of experiment.

for anabolic metabolism, or responds to changes in requirements of other tissues relative to supply, and resulting surplus or deficit, can not be ascertained from net flux measurements alone.

Metabolic impact of nonprotein nitrogen metabolism

Digestion in the ruminant is characterized by extensive recycling of nonprotein N between the lumen of the gut and the liver. Fermentation in the rumen and hindgut produces ammonia, some of which is absorbed into the portal vein and is subsequently removed by the liver, where it is

largely converted to urea. Much as for volatile fatty acids, liver removal of absorbed ammonia is extensive and constant (Reynolds, 1995), such that virtually all the ammonia absorbed into the portal vein is removed, and net total splanchnic flux is typically slightly negative, reflecting removal of ammonia generated in peripheral tissues. For lactating dairy cows and growing cattle, ammonia absorption is typically greater then the net PDV release of α-amino or total amino acid N (for review see Lapierre and Lobley, 2001). Across 315 observations of splanchnic metabolism in growing beef cattle and lactating dairy cows (Firkins and Reynolds, 2005), net PDV release of ammonia N accounted for 42% of N intake on an incremental basis, whilst urea production by the liver accounted for 65%. The magnitude of ammonia absorption in part reflects the nature of the proteins fed, the extent to which ruminal ammonia is utilized for microbial protein synthesis, and the catabolism of urea by microbial urease. Urea released by the liver can be transferred to the lumen of the gut either directly from blood or via saliva. In a recent summarization of published data on the cycling of N between the PDV and liver in ruminants (Lapierre and Lobley, 2001), it was estimated that roughly 2/3 of the urea synthesized in the liver is transferred back to the lumen of the gut. Considering the fate of this urea N in the lumen of the gut, on average 50% was reabsorbed in amino acids synthesized in the rumen, 40% was reabsorbed as ammonia, and 10% was excreted in the feces. This loss of endogenous N would in part reflect microbial protein synthesis in the hindgut, which is influenced by fermentable substrate supply. For example, abomasal starch infusion increased faecal N loss and reduced apparent digestibility of N in late lactation dairy cows (Reynolds *et al*., 2001b), and decreased net PDV absorption of ammonia (Reynolds *et al*., 1998).

The extensive recycling of N between the gut and blood pools represents an evolutionary advantage for animals consuming forages which may under some conditions be limiting in N, but under conditions of N surplus excessive ammonia absorption may also have metabolic disadvantages. In a summarization of measurements of energy metabolism in cattle, Tyrrell *et al.* (1970) noted that cows fed digestible N in excess of calculated requirement had increased heat production that reduced the net availability of energy for production. This was hypothesized to be due to the energy costs of urea synthesis, which on a gross basis is equal to 4 ATP. However, in growing heifers fed isonitrogenous diets differing in forage to concentrate ratio, at equal ME intakes, resulting differences in digested N, ammonia absorption, and liver urea production were not associated with differences in liver oxygen consumption (Reynolds *et al*., 1991a and 1991b). In another study, adding urea at 2% of the DM in a lucerne based diet fed to beef steers markedly increased ammonia absorption and liver urea release, but again had no effect on liver oxygen consumption (Maltby *et al*., 2005a). Other studies in sheep have also found that infusing ammonia into the mesenteric vein of sheep has no significant effect on liver oxygen consumption (see Reynolds, 2005). The failure of increased urea synthesis from ammonia to elicit an increase in oxygen consumption by the liver *in vivo* in part may reflect the fact that the net energy cost of urea synthesis is much lower when the reducing equivalents gained from glutamate transamination and fumarate metabolism are considered (Reynolds *et al*., 1991b; Waterlow, 1999). On an ATP basis, the synthesis of urea from ammonia requires 4 moles of ATP and produces 6 moles of ATP equivalents through NADH generation (Waterlow, 1999). Thus in terms of reducing equivalents required to generate ATP, the increase in urea cycle activity would have a positive net effect. The urea and citric acid cycles are inherently linked, through the flux of both fumarate and α-ketoglutarate. Therefore, the metabolic flexibility of the liver may allow increases in urea cycle

activity with no net cost in terms of oxygen consumption through shifts in the metabolic flux of other metabolites, and the exchange of reducing equivalents.

The lack of an effect of increased ammonia absorption on liver oxygen consumption does not support the hypothesis put forward to explain the effect of excess digestible N intake reported by Tyrrell *et al.* (1970). However, in the studies summarized by Tyrrell *et al.* (1970), excess N was largely fed as protein. In a study in sheep comparing sources of supplemental protein, feeding urea also increased ammonia absorption and liver urea production with no effect on liver oxygen consumption (Ferrell *et al.*, 2001), but feeding isonitrogenous amounts of soybean meal or blood and feather meal increased liver urea production and oxygen consumption. In growing beef steers, adding soybean meal to a 75% concentrate diet increased ammonia absorption and urea production, and increased PDV, liver and body oxygen consumption (Reynolds *et al.*, 1992). These observations suggest that the energy cost of excess protein intake may not be due to the synthesis of urea from ammonia, but may arise as a consequence of the metabolism of amino acids absorbed in excess of requirement, or other metabolic effects of the protein digested (Reynolds, 2005).

In addition to potential energy costs of urea synthesis, it has also been hypothesized that increased ammonia absorption may increase liver catabolism of amino acids (Reynolds, 1992; Parker *et al.*, 1995; Lobley *et al.*, 1995). As one of the two N in urea is derived from glutamate via aspartate, it was proposed that under conditions of excess ammonia supply to the liver the transamination of other amino acids could be required. However, extensive studies in sheep by G. E. Lobley *et al.* (see e.g. Lobley *et al.*, 2000; Milano and Lobley, 2001; Milano *et al.*, 2000) have concluded that even under conditions of extremely high rates of ammonia delivery to the liver there is little, if any, effect on the catabolism of amino acids. Similarly, in maintenance fed steers, feeding urea to increase ammonia absorption had no significant effect on net liver removal of amino acids (Maltby *et al.*, 2005b). Thus it appears that the ruminant liver is capable of metabolizing large amounts of absorbed ammonia to urea, with no apparent adverse metabolic effects in terms of energy or amino acid metabolism. However, the digestion of protein in excess of requirement may incur an energetic cost via increased splanchnic oxygen consumption, although the metabolic origin of this increase in oxidative metabolism is not certain. One possibility is that the response is partly a consequence of the metabolism of branched-chain amino acids in the PDV, or their effects on nutrient metabolism (Layman, 2003).

Conclusions

Without question, the splanchnic tissues are critical determinants of the quantity and profile of amino acids available to peripheral tissues of the ruminant for the production of milk and meat. Not only are they responsible for digestion and absorption of nitrogenous compounds, but they are a major site of amino acid utilization within the body. On the basis of net flux measurements alone, this extensive metabolism of amino acids would appear to reduce the availability of amino acids to other body tissues, and there is no question that many of the critical functions of the splanchnic tissues for maintenance and production impose obligatory costs which impact amino acid supply. However, data from isotopic labeling studies show that with the exception of nonessential amino acids such as glutamate, the majority of the amino acids sequestered by

the portal-drained viscera are derived from the arterial blood pool, and not subject to excessive 'first-pass' metabolism during their absorption. This is logical considering that the PDV is much more than the absorptive enterocytes of the distal small intestine, and contains a large amount of metabolically active tissues, such as those comprising the rumen and hindgut, that have to rely on arterial blood supply to meet their amino acid requirements. Similarly, the majority of the amino acids in the portal vein are derived from arterial blood, thus liver removal of amino acids to a large extent reflects use from the arterial blood pool.

The utilization of amino acids by both the PDV and liver is determined not only by the specific requirements of the splanchnic tissues, but also the availability of amino acids in excess of requirement. Recent data has demonstrated the oxidation of certain essential amino acids by the PDV of pigs and ruminants, and the PDV appears to be particularly important for the utilization of leucine and the other branched chain amino acids, which are not subject to extensive liver metabolism and are often released by the liver on a net basis in lactating dairy cows. There is no question that the utilization of amino acids by splanchnic tissues is integrated with body requirements and sensitive to metabolizable protein supply, but the specific mechanisms by which this regulation occurs are not adequately understood. In addition, there are important interactions between the amount and form of ME supply which impact the productive use of amino acids. For example, abomasal infusion of starch in lactating dairy cows increases body N retention in late lactation (Reynolds *et al*., 2001b). There has been considerable improvement in our understanding of amino acid utilization by splanchnic tissues of ruminants over the course of the last decade, but our ability to predict the efficiency of utilization of specific amino acids will require an accurate prediction of the propensity of the animal for productive use of amino acid supply. There is no question that the efficiencies of utilization of individual amino acids vary with supply and demand, which are integrated with energy metabolism. The efficiency of amino acid use, and the extent of splanchnic catabolism, will undoubtedly vary inversely as metabolizable protein supply changes relative to metabolic demand. The challenge for the refinement of N rationing systems and models of amino acid metabolism will be how best to mathematically represent the considerable metabolic flexibility of amino acid utilization in the splanchnic and other body tissues. As regards the effects of excess protein intake, there appears to be a cost in terms of net energy supply, but the effect is not due to increased ammonia absorption per se, but may be a consequence of other effects of surplus metabolizable protein supply.

References

AFRC (1998). Response in the Yield of Milk Constituents to the Intake of Nutrients by Dairy Cows. Technical Committee on Responses to Nutrients, Report No. 11. CABI Publishing, Wallingford, 96pp.

Aikman, P.C., C.K. Reynolds, D.J. Humphries, D.E. Beever & J.C. MacRae (2002). Milk protein response to abomasal or mesenteric vein essential amino acid infusion in lactating dairy cows. Journal of Dairy Science, 85, 1079-1084.

Bergman, E.N. & R.N. Heitmann 1980. Integration of whole-body amino acid metabolism. In: Buttery, P. J. & Lindsay, D. B. (eds.) Protein Deposition in Animals. Butterworth & Co, London, UK. pp. 69-84.

Berthiaume, R., P. Dubreuil, M. Stevenson, B.W. McBride & H. Lapierre (2001). Intestinal disappearance and mesenteric and portal appearance of amino acids in dairy cows fed ruminally protected methionine. Journal of Dairy Science, 84, 194-203.

Blouin, J.P., J.F. Bernier, C.K. Reynolds, G.E. Lobley, P. Dubreuil & H. Lapierre (2002). Effect of diet quality on splanchnic fluxes of nutrients and hormones in lactating dairy cows. Journal of Dairy Science, 85, 2618-2630.

Bos, C., B. Stoll, H. Fouillet, C. Gaudichon, X.F.Guan, M.A. Grusak, P.J. Reeds, D. Tome & D.G. Burrin (2003). Intestinal lysine metabolism is driven by the enteral availability of dietary lysine in piglets fed a bolus meal. American Journal of Physiology, 285, E1246-E1257.

Bruckenthal, I., G.B. Huntington, C.K. Baer & R.A. Erdman (1997). The effect of abomasal infusion of casein and recombinant somatotropin hormone injection on nitrogen balance and amino acid fluxes in portal-drained viscera and net hepatic and total splanchnic blood in Holstein steers. Journal of Animal Science, 75, 1119-1129.

Doepel, L., D. Pacheco, J.J. Kennelly, M.D. Hanigan, I.F. Lopez & H. Lapierre (2004). Milk protein synthesis as a function of amino acid supply. Journal of Dairy Science, 87, 1279-1287.

Elwyn, D.H. (1970). The role of the liver in regulation of amino acid and protein metabolism. In: H.N. Munro (ed.) Mammalian Protein Metabolism, Vol. 4. Academic Press, New York, pp. 523-557.

Fan, M.Z. & W.C. Sauer (2002). Determination of true ileal amino acid digestibility and the endogenous amino acid outputs associated with barley samples for growing-finishing pigs by the regression analysis technique Journal of Animal Science, 80, 1593-1605.

Ferrell, C.L., H.C. Freetly, A.L. Goetsch & K.K. Kreikemeier (2001). The effect of dietary nitrogen and protein on feed intake, nutrient digestibility, and nitrogen flux across the portal-drained viscera and liver of sheep consuming high-concentrate diets ad libitum. Journal of Animal Science, 79, 1322-1328.

Firkins, J.L. & C.K. Reynolds (2005). Whole Animal Nitrogen Balance in Cattle. In, E. Pfeffer & A. N. Hristov (eds.), Nitrogen and Phosphorus Nutrition of Cattle and the Environment. CAB International, in press.

Fuller, M.F. & P.J. Reeds (1998). Nitrogen recycling in the gut. Annual Review of Nutrition, 18, 385-411.

Gibb, M.J., W.E. Ivings, M.S. Dhanoa & J.D. Sutton (1992). Changes in body components of autumn-calving Holstein-Friesian cows over the first 29 weeks of lactation. Animal Production, 55, 339-360.

Hanigan, M.D., L.A. Crompton, C.K. Reynolds, D. Wray-Cahen, M.A. Lomax & J. France (2004a). A model of liver nitrogen metabolism in the lactating dairy cow. The Journal of Theoretical Biology, 228, 271-289.

Hanigan, M.D., C.K. Reynolds, D.J. Humphries, B. Lupoli & J.D. Sutton (2004b). A model of net amino acid absorption and utilization by the portal-drained viscera of the cow. Journal of Dairy Science, 87, 4247-4268.

Huntington, G.B., J.H. Eisemann & J.M. Whitt (1990). Portal blood flow in beef steers: comparison of techniques and relation to hepatic blood flow, cardiac output and oxygen uptake. Journal of Animal Science, 68, 1666-1673.

Lapierre, H. & G.E. Lobley (2001). Nitrogen recycling in the ruminant: a review. Journal of Dairy Science 84 (E. Suppl.), E223-E236.

Lapierre, H., C.K. Reynolds, T.H. Elsasser, P. Gaudreau, P. Brazeau & H.F. Tyrrell (1992). Effects of growth hormone releasing factor and intake on energy metabolism in growing beef steers: Net hormone metabolism by portal drained viscera and liver. Journal of Animal Science, 70, 742-751.

Layman, D.K. (2003). The role of leucine in weight loss diets and glucose homeostasis. Journal of Nutrition, 133, 261S-267S.

Lien, K.A., W.C. Sauer & M.E. Dugan (1997). Evaluation of the 15N-isotope dilution technique for determining the recovery of endogenous protein in ileal digesta of pigs: effect of the pattern of blood sampling, precursor pools, and isotope dilution technique. Journal of Animal Science, 75, 159-169.

Lindsay, D.B. & C.K. Reynolds (2005). Metabolism of the Portal-Drained Viscera. In: J. Dykstra, J. France & M. Forbes (eds.), Quantitative Aspects of Ruminant Digestion & Metabolism, Second Edition, CAB International, in press.

Lobley, G.E., D.M. Bremner & D.S. Brown (2001). Response in hepatic removal of amino acids by the sheep to short-term infusions of varied amounts of an amino acid mixture into the mesenteric vein. British Journal of Nutrition, 85, 689-698.

Lobley, G.E., A. Connell, M.A. Lomax, D.S. Brown, E. Milne, A.G. Calder & D.A.H. Farringham (1995). Hepatic detoxification of ammonia in the ovine liver: possible consequences for amino acid catabolism. British Journal of Nutrition, 75, 217-235.

Lobley, G.E., G.D. Milano & J.G. van der Walt (2000). The liver: integrator of nitrogen metabolism. In: Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. Cronje, P.B. (ed.). CAB International, Wallingford, UK. pp. 149-168.

Lobley, G.E., X. Shen, G. Le, D.M. Bremmer, E. Milne, A.G. Calder, S.E. Anderson & N. Dennison (2003). Oxidation of essential amino acids by the ovine gastro-intestinal tract. British Journal of Nutrition, 89, 617-629.

MacRae, J.C., P.J. Buttery & D.E. Beever (1988). Nutrient interactions in the dairy cow. In: P.C. Garnsworthy (ed.) Nutrition and Lactation in the Dairy Cow. Butterworths, London, pp. 55-75.

MacRae, J.C., L.A. Bruce, D.S. Brown & A.G. Calder (1997a). Amino acid use by the gastrointestinal tract of sheep given lucerne forage. American Journal of Physiology, 273, G1200-G1207.

MacRae, J.C., L.A. Bruce, D.S. Brown, D.A.H. Farningham & M. Franklin (1997b). Absorption of amino acids from the intestine and their net flux across the mesenteric- and portal-drained viscera of lambs. Journal of Animal Science, 75, 3307-3314.

MacRae, J.C., P.J. Buttery & D.E. Beever (1988). Nutrient interactions in the dairy cow. In: P.C. Garnsworthy (ed.) Nutrition and Lactation in the Dairy Cow. Butterworths, London, pp. 55-75.

Maltby, S.A., C.K. Reynolds, M.A. Lomax & D.E. Beever (2005a). Splanchnic metabolism of non-nitrogenous nutrients and hormones in steers fed alfalfa under conditions of increased net absorption of ammonia and L-arginine across the portal-drained viscera. Journal of Animal Science, 83, 1088-1096.

Maltby, S.A., C.K. Reynolds, M.A. Lomax & D.E. Beever (2005b). Splanchnic metabolism of nitrogenous nutrients and urinary nitrogen excretion in steers fed alfalfa under conditions of increased net absorption of ammonia and L-arginine across the portal-drained viscera. Journal of Animal Science, 83, 1075-1087.

Milano, G.D., A. Hotston-Moore & G.E. Lobley (2000). Influence of hepatic ammonia removal on ureagenesis, amino acid utilization and energy metabolism in the ovine liver. British Journal of Nutrition, 83, 307-315.

Milano, G.D. & G.E. Lobley (2001). Liver nitrogen movements during short-term infusion of high levels of ammonia into the mesenteric vein of sheep. British Journal of Nutrition, 86, 507-513.

NRC (2001). Nutrient Requirements of Dairy Cattle. 7th Revised Edition. National Academy Press, Washington, DC.

Ouellet, D.R., M. Demers, G. Zuur, G.E. Lobley, J.R. Seoane, J.V. Nolan & H. Lapierre (2002). Effect of dietary fiber on endogenous nitrogen flows in lactating dairy cows. Journal of Dairy Science, 85, 3013-3025.

Parker, D.S., M.A. Lomax, C.J. Seal & J.C. Wilton (1995). Metabolic implications of ammonia production in the ruminant. Proceedings of the Nutrition Society, 54, 549-563.

Rémond, D., L. Bernard, B. Chauveau, P. Nozière & C. Poncet (2003). Digestion and nutrient net fluxes across the rumen, and the mesenteric- and portal-drained viscera in sheep fed with fresh forage twice daily: net balance and dynamic aspects. British Journal of Nutrition, 89, 649-666.

Rerat, A., J. Jung & J. Kande (1988). Absorption kinetics of dietary hydrolysis products in conscious pigs given diets with different amounts of fish protein. 2. Individual amino acids. British Journal of Nutrition, 60, 105-120.

Reynolds, C.K. (1992). Nitrogen metabolism by ruminant liver. Journal of Nutrition, 122, 850-854.

Reynolds, C.K. (1995). Quantitative aspects of liver metabolism in ruminants. In: W. v. Engelhardt, S. Leonhard-Marek, G. Breves & D. Giesecke (eds.) Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction: Proceedings of the Eighth International Symposium on Ruminant Physiology. Ferdinand Enke Verlag, Stuttgart, Germany, pp. 351-372.

Reynolds, C.K. (2002). Economics of visceral tissue metabolism in ruminants. Toll keeping or internal revenue service? Journal of Animal Science, 80 (Electronic Supplement), E74-E84.

Reynolds, C.K. (2005) Nitrogen metabolism by splanchnic tissues of ruminants. In: D. Burrin & H. Merssman (eds.), Biology of Metabolism of Growing Animals, Elsevier Science, Oxford, England, 2005, 197-220.

Reynolds, C.K., P.C. Aikman, B. Lupoli, D.J. Humphries & D.E. Beever (2003). Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. Journal of Dairy Science, 86, 1201-1217.

Reynolds, C.K., B.J. Bequette, J.S. Caton, D.J.Humphries, P.C. Aikman, B. Lupoli, B. & J.D. Sutton (2001a). Effects of intake and lactation on absorption and metabolism of leucine and phenylalanine by splanchnic tissues of dairy cows. Journal of Dairy Science, 84 (Supplement 1), 362.

Reynolds, C.K., S.B. Cammell, D.J. Humphries, D.E. Beever, J.D. Sutton & J.R. Newbold (2001b). Effects of post-rumen starch infusion on milk production and energy metabolism in dairy cows. Journal of Dairy Science, 84, 2250-2259.

Reynolds, C.K., D.P. Casper, D.L. Harmon & C.T. Milton (1992). Effect of CP and ME intake on visceral nutrient metabolism in beef steers. Journal of Animal Science, 70 (Supplement 1), 315.

Reynolds, C.K., D.L. Harmon, R.L. Prior, D.P. Casper & C.T. Milton (1995). Splanchnic metabolism of amino acids in beef steers fed diets differing in CP content at two ME intakes. Journal of Animal Science, 73 (Supplement 1), 270.

Reynolds, C.K., D.L. Harmon, R.L. Prior & H.F. Tyrrell (1994). Effects of mesenteric vein L-alanine infusion on liver metabolism of organic acids by beef heifers fed diets differing in forage-to-concentrate ratio. Journal of Animal Science, 72, 3196-3206.

Reynolds, C.K., D.J. Humphries, S.B. Cammell, J. Benson, J.D. Sutton & D.E. Beever (1998). Effects of abomasal wheat starch infusion on splanchnic metabolism and energy balance of lactating dairy cows. In: K.J. McCracken, E.F. Unsworth & A.R.G. Wylie (eds.), Energy Metabolism of Farm Animals, Proceedings of the 14th Symposium on Energy Metabolism. CAB International, Wallingford, UK. pp. 39-42.

Reynolds, C.K., B. Lupoli, P.C. Aikman, D.J. Humphries, L.A. Crompton, J.D. Sutton, J. France, D.E. Beever & J.C. MacRae (1999). Effects of abomasal casein or essential amino acid infusions on splanchnic metabolism in lactating dairy cows. Journal of Animal Science, 77 (Supplement 1), 266.

Reynolds, C.K., H.F. Tyrrell & P.J. Reynolds (1991a). Effects of diet forage-to-concentrate ratio and intake on energy metabolism in growing beef heifers: Whole body energy and nitrogen balance and visceral heat production. Journal of Nutrition, 121, 994-1003.

Reynolds, C.K., H.F. Tyrrell & P.J. Reynolds (1991b). Effects of diet forage-to-concentrate ratio and intake on energy metabolism in growing beef heifers: Net nutrient metabolism by visceral tissues. Journal of Nutrition, 121, 1004-1015.

Stoll, B., J. Henry, P.J. Reeds, H. Yu, F. Jahoor & D.G. Burrin (1998). Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. Journal of Nutrition, 128, 606-614.

Stoll, B., D.G. Burrin, J. Henry, H. Yu, F. Jahoor & P.J. Reeds (1999). Substrate oxidation by the portal drained viscera of fed piglets. American Journal of Physiology, 277, E168-E175.

Sutton, J.D. & C.K. Reynolds (2003). Digestion and Absorption of Nutrients in the Small Intestine of Lactating Ruminants. In: H. Roginski, P. Fox & J. Fuquay (eds.), Encyclopedia of Dairy Sciences, Academic Press, San Diego, pp. 2120-2127.

Tagari, H. & E.N. Bergman (1978). Intestinal disappearance and portal blood appearance of amino acids in sheep. Journal of Nutrition, 108, 790-803.

Tyrrell, H.F., P.W. Moe & W.P. Flatt (1970). Influence of excess protein intake on energy metabolism of the dairy cow. In: Schurch, A. & Wenks, C. (eds.) Energy Metabolism of Farm Animals. pp. 69–72. EAAP Publ. No. 13. Juris Verlag, Zurich, Switzerland. pp. 69–72.

Van der Schoor, S.R.D., J.B. Van Goudoever, B. Stoll, J.F. Henry, J.R. Rosenberger, D.G. Burrin & P.J. Reeds (2001). The pattern of intestinal substrate oxidation is altered by protein restriction in pigs. Gastroenterology, 121, 1167-1175.

Van Goudoever, J.B., B. Stoll, J.F. Henry, D.B. Burrin & P.J. Reeds (2000). Adaptive regulation of intestinal lysine metabolism. Proceedings Of The National Academy Of Sciences Of The United States Of America, 97, 11620-11625

Waterlow, J.C. (1999). The mysteries of nitrogen balance. Nutr. Res. Rev. 12:25-54.

Whitelaw, F.G., J.S. Milne, E.R. Ørskov & J.S. Smith (1988). The nitrogen and energy metabolism of lactating cows given abomasal infusions of casein. British Journal of Nutrition, 55, 537-556.

Windmueller, H.G. & A.E. Spaeth (1980). Respiratory fuels and nitrogen metabolism in vivo in small intestine of rats. Quantitative importance of glutamine, glutamate and aspartate. Journal of Biological Chemistry, 255, 107-112.

Wray-Cahen, D., J.A. Metcalf, F.R.C. Backwell, B.J. Bequette, D.S. Brown, J.D. Sutton & G.E. Lobley (1997). Hepatic response to increased exogenous supply of plasma amino acids by infusion into the mesenteric vein of Holstein-Friesian cows in late gestation. British Journal of Nutrition 78:913-930.

Yu, F., L.A. Bruce, A.G. Calder, E. Milne, R.L. Coop, F. Jackson, G.W. Horgan & J.C. MacRae (2000). Subclinical infection with the nematode *Trichostrongylus colubriformis* increases gastrointestinal tract leucine metabolism and reduces availability of leucine for other tissues. Journal of Animal Science, 78, 380-390.

Splanchnic metabolism of short-chain fatty acids in the ruminant

N.B. Kristensen[1] and D.L. Harmon[2]
[1]Danish Institute of Agricultural Sciences, Department of Animal Nutrition and Physiology, DK-8830 Tjele, Denmark
[2]University of Kentucky, Department of Animal Sciences, Lexington, KY 40546, USA

Abstract

The unique digestive system of ruminants has major impacts on their energy metabolism because carbohydrates are largely fermented and only to a small extent digested by host enzymes. Short-chain fatty acids (SCFA) are produced during fermentation in the ruminant forestomachs and quantitatively SCFA are the most important substrates for ruminant energy metabolism. The central dogma of ruminant nutritional physiology has been that gut epithelia have a large metabolism of SCFA and that SCFA are available to other tissues in considerably lower amounts than ruminal production would suggest. A series of papers published since the IX ISRP question the traditional view on SCFA metabolism and it is concluded that the forestomach epithelia contrary to our previous understanding, do not metabolize extensive amounts of acetate and propionate. The forestomachs of ruminants have a high affinity for butyrate and valerate and the physiology of gut epithelial and hepatic metabolism of SCFA is discussed in this context.

Keywords: short-chain fatty acids, ruminal epithelium, liver, metabolism

Introduction

Because of extensive anaerobic fermentation in the forestomachs of ruminants, short-chain fatty acids (SCFA) and not carbohydrates are the quantitatively most important substrates for ruminant energy metabolism. Studies of SCFA production based on intraruminal isotopic dilution consistently agree on a large contribution of SCFA to ruminant energy metabolism although variable results have been obtained. Estimates on the contribution of SCFA to DE have been found in the range from 20 to 65% of DE (Sutton, 1985; Bergman, 1990). On top of this variability, that probably to some extent is caused by inherent technical problems in methodology (Sutton and Morant, 1978), arises the problem to account for the metabolism of SCFA in body tissues. One of the central dogmas of ruminant nutritional physiology has been that 30 to 90% of all SCFA absorbed across the ruminal epithelium are metabolized before entering the portal vein (Bergman and Wolff, 1971). Numerous reviews have come to the same conclusion that the ruminal epithelium had a unique role in ruminant energy metabolism by its huge SCFA metabolism (Bergman, 1990; Britton and Krehbiel, 1993; Seal and Reynolds, 1993; Rémond *et al*., 1995; Kristensen *et al*., 1998; Seal and Parker, 2000). However, until recently, most of the work done to relate intraluminal SCFA production to portal SCFA absorption has considered SCFA to be end products of microbial metabolism whose only disappearance, other than absorption (including SCFA absorbed distal to the rumen), would be from conversion into other SCFA (i.e. acetate - butyrate inter conversion). Based on the hypothesis that rumen microbial metabolism of SCFA might impact the relationship between apparent ruminal (GIT) production of SCFA and subsequent measures of portal absorption, a number of recent studies have attempted to separate

animal and microbial pathways of SCFA metabolism by use of the washed rumen technique as well as by intraruminal metabolism of isotopic labeled acetate. The aim of the present review is to evaluate the impact of these new studies on our understanding of ruminant SCFA metabolism.

Low first pass sequestration of acetate and propionate by ruminal epithelium

To address the availability of SCFA to ruminants the two experimental models most frequently used have been the intraruminal isotope dilution technique (Sutton *et al*., 2003) and net flux measurements in multicatheterized animals (Seal and Reynolds, 1993). The crux of the controversy is that the two techniques usually have given widely different results as first observed in the classic paper of Bergman and Wolff (1971), and that the difference between these two experimental approaches has been interpreted as mucosal metabolism of SCFA during absorption. However, a number of concerns have been raised (Kristensen and Danfær, 2001) as to how a tissue bed as limited in mass as the forestomach epithelia could possibly have such a high energy metabolism as previously suggested (Bergman and Wolff, 1971). This dilemma has triggered interest in revisiting the problem of accounting for SCFA metabolism in the ruminant.

In order to separate rumen microbial and animal metabolism of SCFA the temporarily washed rumen technique has been used in multicatheterized sheep and steers. Using this technique, a precisely defined amount of SCFA in a buffer solution can be added to the rumen and a stable rate of SCFA absorption can be maintained for hours by continuous infusion of SCFA into the rumen. Simultaneously, the permanent indwelling catheters in major splanchnic blood vessels (Huntington *et al*., 1989; Seal and Reynolds, 1993), in combination with systemic infusion of [^{13}C]-labeled acetate, allows for measurement of the unidirectional influx of acetate to the portal blood [after correction for portal-drained visceral uptake of arterial acetate (Kristensen *et al*., 1996)] and hepatic metabolism of the absorbed SCFA. The washed rumen approach is related to the intragastric nutrition technique (Ørskov *et al*., 1984) when applied in multicatheterized animals (Gross *et al*., 1990), but the washed rumen technique is of short duration, usually less than 8 to 10h, and it prevents the animal from adapting to artificial nutritional conditions.

In sheep with a functional rumen, 54 to 62% of acetate, propionate and isobutyrate infused into the rumen were recovered in the portal vein (Table 1). However, with the washed rumen technique, acetate and isobutyrate were fully accounted for in the portal vein with only 5 to 10% of propionate apparently metabolized during absorption. The difference between these experimental techniques indicates that the extent to which acetate and propionate are metabolized in the ruminal epithelium is less than hitherto assumed.

The portal vein recovery of acetate increased from 54% to more than 100% of the ruminal absorption rate in sheep (Kristensen *et al*., 2000a) and in steers (Kristensen and Harmon, 2004c). The difference in results with the differing experimental approaches indicates that the ruminal microbes, under usual feeding conditions, metabolize a relatively large fraction of the acetate produced or infused into the rumen. However, the recoveries of 105 to 109% could also indicate that either the corrections for portal-drained visceral (PDV) uptake of arterial acetate (second pass sequestration measured by intravenous infusion of [2-^{13}C]acetate) are overestimating the PDV uptake of arterial acetate or that there is endogenous acetate production by ruminal epithelium

Table 1. Portal vein recovery (%) of SCFA infused into a functional rumen of sheep or into the washed rumen of sheep or steers.

	Functional rumen	Washed rumen	
Item	**Sheep**	**Sheep**	**Steer**
Acetate[1]	54 ± 8[2]	109 ± 7[4]	105 ± 3[5]
Propionate	62 ± 7[2]	95 ± 7[4]	91 ± 2[5]
Isobutyrate	60 ± 3[3]	102 ± 9[4]	101 ± 9[6]
Butyrate	11 - 25 ± 2[2]	23 ± 3[4]	18 - 52 ± 4[6]
Isovalerate	-	48 ± 5[4]	54 ± 4[5]
Valerate	14 - 31 ± 2[3]	32 ± 4[4]	16 - 54 ± 3[6]
Caproate	-	-	54 ± 2[7]
Heptanoate	-	-	43 ± 2[7]

[1]Values for acetate are corrected for PDV uptake of arterial acetate using systemic infusion of [2-^{13}C]acetate.

[2](Kristensen *et al*., 1996), [3](Kristensen *et al*., 2000b), [4](Kristensen *et al*., 2000a), [5](Kristensen and Harmon, 2004c), [6](Kristensen and Harmon, 2004a), [7](Kristensen and Harmon, 2004b)

and/or other PDV tissues. If the ruminal epithelium synthesizes acetate *in vivo*, this would be in agreement with *in vitro* observations that ruminal epithelium mounted in Ussing chambers produce acetate (Sehested *et al*., 1999). However, it is important to consider that although the ruminal epithelium might not take up acetate, the remaining PDV tissues remove acetate from the arterial blood (Kristensen and Harmon, 2004c). This 'second pass' uptake could account for ~30% of the acetate absorbed from the rumen. Therefore, acetate is assumed to be a substrate of major quantitative importance for the extensive oxidative metabolism (Huntington, 1990) of PDV tissues other than the ruminal epithelium.

The portal recovery of propionate was 91 to 95% in the washed rumen experiments in sheep and steers, indicating that the ruminal epithelium metabolizes some propionate during absorption. The major products of propionate metabolism in the ruminal epithelium are expected to be CO_2 and lactate. The net flux of lactate across the PDV increased with SCFA buffer in the rumen compared to the buffer without SCFA, and could at a maximum account for ~15% of the propionate absorption from the rumen. However, metabolism of valerate by the rumen epithelium will also produce propionyl-CoA for lactate production and the PDV net absorption of lactate can therefore originate from at least three substrates: glucose (glycolysis), propionate and valerate metabolism. Also, previous studies have shown that the metabolism of propionate into lactate by the ruminal epithelium (Weigand *et al*., 1972) is substantially lower (< 5%) *in vivo* compared with *in vitro* observations (Weigand *et al*., 1975). Weekes and Webster (1975) observed that intraruminally infused propionate did not lead to substantial increase in lactate production by the PDV. Collectively, these results suggest a relatively small contribution of propionate carbon to PDV net release of lactate.

It can be concluded that when SCFA absorption was studied after removal of the rumen microbial ecosystem, all ruminally absorbed acetate and isobutyrate were recovered in the portal vein (no

first pass uptake) and only 5 - 10% of the absorbed propionate was metabolized during absorption. Portal-drained visceral uptake of arterial acetate (second pass uptake) implies that net absorption into the portal vein accounts for ~70% of the acetate absorbed, with the remainder metabolized by PDV tissues other than the ruminal epithelium.

Large first pass sequestration of butyrate and valerate by the ruminal epithelium

Experiments with functional and washed rumens have both shown that butyrate and valerate are extensively metabolized prior to absorption into the portal vein (Table 1). Thus, it does not appear that rumen microbial metabolism is responsible for the low recoveries of ruminal butyrate and valerate, but rather that these SCFA are metabolized by the ruminal epithelium. Extensive butyrate metabolism during absorption is a phenomenon observed in other species and butyrate is generally considered a unique metabolite for gut epithelia (Topping and Clifton, 2001).

Early studies (Pennington, 1952) showed that the ruminal epithelium produces substantial amounts of ketone bodies from butyrate metabolism. Valerate, a 5-carbon fatty acid, is metabolized to acetyl-CoA and propionyl-CoA by β-oxidation. Therefore, valerate can be both glucogenic and ketogenic. *In vitro* incubation of rumen epithelium with valerate produces both 3-hydroxybutyrate and lactate (Weigand *et al*., 1975). The ruminal epithelium, therefore requires a propionyl-CoA pathway, even if no propionate is metabolized. The propionyl-CoA sink in the ruminal epithelium is probably not gluconeogenesis as in the liver, but the oxidative decarboxylation of malate to form pyruvate by malic enzyme (EC 1.1.1.40; Young *et al*., 1969). Pyruvate can be reduced to lactate before being released into the blood.

Although ketogenesis is expected to be a primary pathway for butyrate metabolism in the ruminal epithelium, the net portal flux of ketones accounts for only ~25% of absorbed butyrate (Krehbiel *et al*., 1992; Kristensen *et al*., 1996). However, for 3-hydroxybutyrate, as for acetate, the PDV uptake from arterial blood can have a major impact on its net portal fluxes. In sheep, up to 15% of the arterial supplied 3-hydroxybutyrate was taken up in PDV tissues (Kristensen *et al*., 2000c). Because of the small portal-arterial concentration difference relative to the arterial concentration of 3-hydroxybutyrate this relatively small fractional uptake of arterial 3-hydroxybutyrate in PDV tissues has a large impact on the estimated 3-hydroxybutyrate production in gut epithelia. Thus, correction for PDV uptake of arterial 3-hydroxybutyrate doubles the estimated 3-hydroxybutyrate production in the PDV and the corrected PDV production rate of 3-hydroxybutyrate could account for ~40% of the butyrate absorption. Although PDV second pass uptake of 3-hydroxybutyrate has not been determined in cattle we expect similar figures compared with sheep because of the negative mesenteric-drained visceral flux of 3-hydroxybutyrate observed in cattle (Reynolds and Huntington, 1988).

In vitro, the ruminal epithelium has the capacity to metabolize a broad range of fatty acids including medium- and long-chain fatty acids (Hird *et al*., 1966; Jesse *et al*., 1992). However, little information is available on the *in vivo* uptake and metabolism of fatty acids longer than the SCFA. Data from the washed rumen model suggests that the fraction of the fatty acids metabolized decreases with a chain length exceeding that of valerate (Kristensen and Harmon, 2004b). At this

point, the available data suggests that the metabolic window of the rumen epithelium under *in vivo* conditions is narrow and centered around metabolism of butyrate and valerate. Specifically, acetate and propionate have much lower fractional uptakes.

Is the ruminal epithelium a specialized butyrate scavenger?

Butyrate is generally considered a unique metabolite for gut epithelial function (Topping and Clifton, 2001). One way to explain the special behavior of gut epithelia towards butyrate compared with acetate and propionate is that butyrate is important as an energy source for epithelial cells (Bugaut, 1987). However, even though butyrate is fulfilling a significant part of the oxidative needs of the epithelial cells it does not adequately explain why butyrate is favored as substrate for epithelial energy metabolism. Complete oxidation of butyrate necessitates its catabolism to acetyl-CoA for entry into the TCA cycle. But, why does the gut prefer butyrate when acetate is available in greater quantities to fill this need. We could speculate that the reason the gut epithelia metabolizes butyrate is not only to harvest acetyl-CoA units' and produce ketones, but also to serve a function for the whole organism.

It is obvious that butyrate is handled differently from acetate and propionate by the epithelia (Pennington, 1952), but another question remains to be answered: is butyrate a unique metabolite? Valerate, for example, is also efficiently metabolized by the rumen epithelium (Table 1) and it has been shown that the epithelium has the capacity to metabolize medium-chain (Hird *et al*., 1966; Kristensen and Harmon, 2004b), as well as long-chain fatty acids (Jesse *et al*., 1992).

Butyrate is an important substrate for gut epithelia, compared with acetate and propionate but apparently it is not a unique nutrient. Acetate, propionate, and isobutyrate are all end-products of endogenous pathways in the animal. Acetate has the lowest membrane permeability, is utilized from peripheral blood in extra hepatic tissues (Pethick and Lindsay, 1982), and is a universal metabolite in the body in the form of acetyl-CoA. Propionate is the main source of 3-carbon units for gluconeogenesis in the ruminant liver and it is efficiently removed by the liver (Leng and Annison, 1963). The endogenous sources of propionate include degradation of odd chained fatty acids and some amino acids (methionine, threonine, isoleucine, and valine). Isobutyrate (an intermediate from catabolism of valine) appears in relatively low concentrations in the rumen, but it is also efficiently removed by the liver for gluconeogenesis (Stangassinger and Giesecke, 1979). These SCFA are not only well-tolerated in hepatic and peripheral tissues, but they are key intermediates (especially acetate and propionate) in the metabolism and this agrees with a limited uptake of these SCFA in the gut epithelia.

Butyrate, valerate, and medium-chain fatty acids (MCFA) are less polar compared with SCFA with a shorter carbon chain and will have a relatively high permeability through cell membranes. One way of controlling permeability is by partial oxidation of these SCFA into acetoacetate and 3-hydroxybutyrate in the gut epithelia. When butyrate appears in the systemic circulation or is added to cell cultures, it has been shown to have a number of effects: inhibition of growth and induction of morphological changes in cultured cells of different origins including ruminal epithelial cell lines (Prasad and Sinha, 1976; Gálfi *et al*., 1991); being an insulin secretagogue (Manns and Boda, 1967) inhibition of gastrointestinal motility by stimulation of epithelial

receptors (Crichlow, 1988) and/or via systemic effects (Le Bars *et al*., 1954); stimulation of rumen epithelial development (Sander *et al*., 1959); or killing (2.5 mmol butyrate / kg BW in lambs) the animal (Manns and Boda, 1967).

The epithelia of the gut have apparently evolved to perform gate keeping functions by controlling the entry of butyrate and longer chain fermentation acids. It is tempting to speculate that the side effects of these gate keeping functions are that these acids become quantitatively important oxidative substrates for the gut epithelia.

Hepatic SCFA metabolism

The liver and ruminal epithelium have different affinities towards individual SCFA. All SCFA, except acetate, are handled differently in these two tissues and this impacts the relative tissue uptake of the SCFA. The low rumen epithelial affinity but high liver affinity for propionate ensures that the largest propionate metabolism occurs in the liver whereas, by contrast, butyrate is removed substantially by the ruminal epithelium prior to reaching the liver (Figure 1).

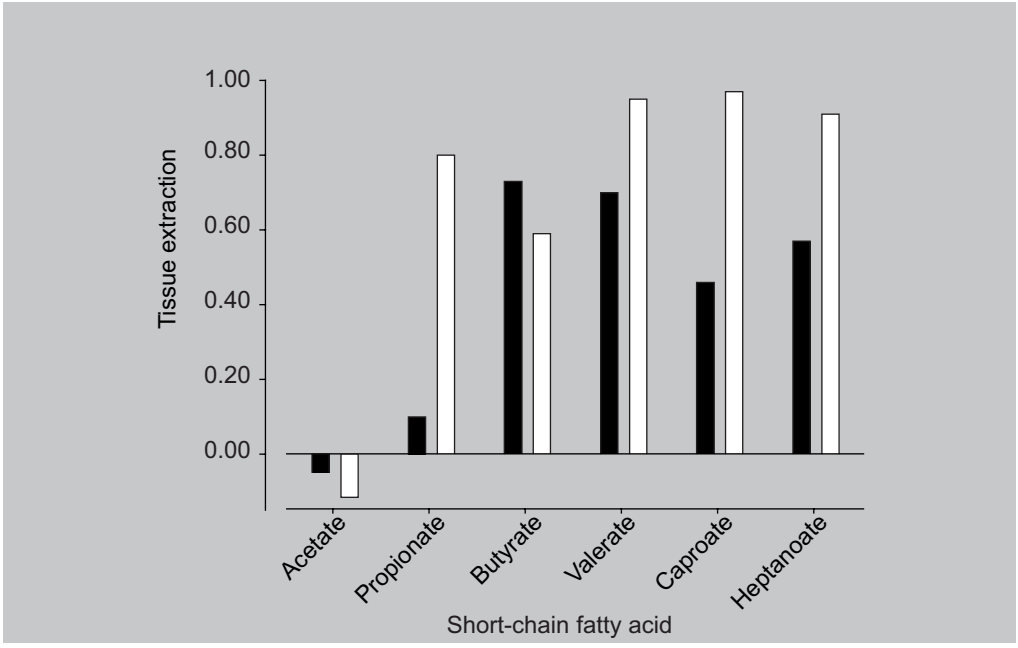

Figure 1. Fractional extraction of short-chain fatty acids in ruminal epithelium (closed bar) and liver (open bar). Data from washed rumen experiments with steers (Kristensen and Harmon, 2004c; Kristensen and Harmon, 2004a; Kristensen andHarmon, 2004b).

Acetate

On a net flux basis acetate is the only SCFA that is not cleared from the blood on passage through the liver. Indeed, more acetate is produced in the liver than removed, leading to net production of acetate in the liver (Reynolds, 1995). However, in both sheep and cattle there is a small unidirectional uptake of acetate by the liver (Bergman and Wolff, 1971; Kristensen and Harmon, 2004c) which is offset by a greater unidirectional production of acetate that leads to net production by the liver. Released acetate derives from the acetyl-CoA pool after hydrolysis of the thioester by acetyl-CoA hydrolase (EC 3.1.2.1). This enzyme is widely distributed in sheep tissues with the highest activity found in liver (Knowles *et al*., 1974). To our knowledge there is no clear picture of the precursors for hepatic acetate production. It is generally assumed that hepatic uptake of nonesterified fatty acids as well as fatty acids from triglycerides serve as precursors for 3-hydroxybutyrate as well as for acetate released by the liver (Bell, 1980). Further, a large portion of acetate released by the ruminant liver originates from peroxisomal β-oxidation. The acetyl-CoA from peroxisomal β-oxidation would not be directly available for mitochondrial metabolism (Van den Bosch *et al*., 1992) and because the ruminant liver has no, or limited, de novo synthesis of fatty acids from cytosolic acetyl-CoA, this pool of acetyl-CoA may provide for acetate production. If peroxisomal β-oxidation is the source for hepatic acetate we would expect that very-long chain fatty acids (C20 - C24), branched fatty acids and polyunsaturated fatty acids would be quantitatively the largest contributors to acetate production (Van den Bosch *et al*., 1992). Ethanol from silage could also be oxidized to acetate in the liver.

Propionate

Propionate is the major glucogenic substrate in fed ruminants (Danfær *et al*., 1995) and the liver has both a high affinity and high capacity for metabolism of propionate (Berthelot *et al*., 2002). In dairy cows, the liver removes ~94% of the net portal flux of propionate. However, the net splanchnic flux of propionate will increase when propionate supply is increased in the portal vein (Berthelot *et al*., 2002; Majdoub *et al*., 2003).

Increased butyrate load to the liver reduces the hepatic extraction of propionate *in vitro* (Aiello *et al*., 1989) and *in vivo* at least in experiments where these have been infused for short durations (i.e. less than 24h; Kristensen and Harmon, 2004a). In experiments with steers it was found that increased ruminal butyrate absorption increased the splanchnic propionate release from 8 to 22% of the ruminal absorption rate (Kristensen and Harmon, 2004a). Sudden increased butyrate absorption might therefore be ketogenic by providing substrate for ketogenesis and probably as well by shifting propionate metabolism from the liver to peripheral tissues.

Hepatic glucose output generally correlates with feed intake (Reynolds, 1995) and milk production (Danfær, 1994) so it is not surprising that hepatic propionate uptake and glucose output are strongly correlated (Figure 2) when evaluated in cattle of different size and levels of feed intake.

Although propionate uptake and glucose output by the liver is correlated when evaluated across a broad range of daily feed intakes, it does not imply that propionate per se drives glucose production and turnover in ruminants. When propionate availability is manipulated (by feeding or infusion),

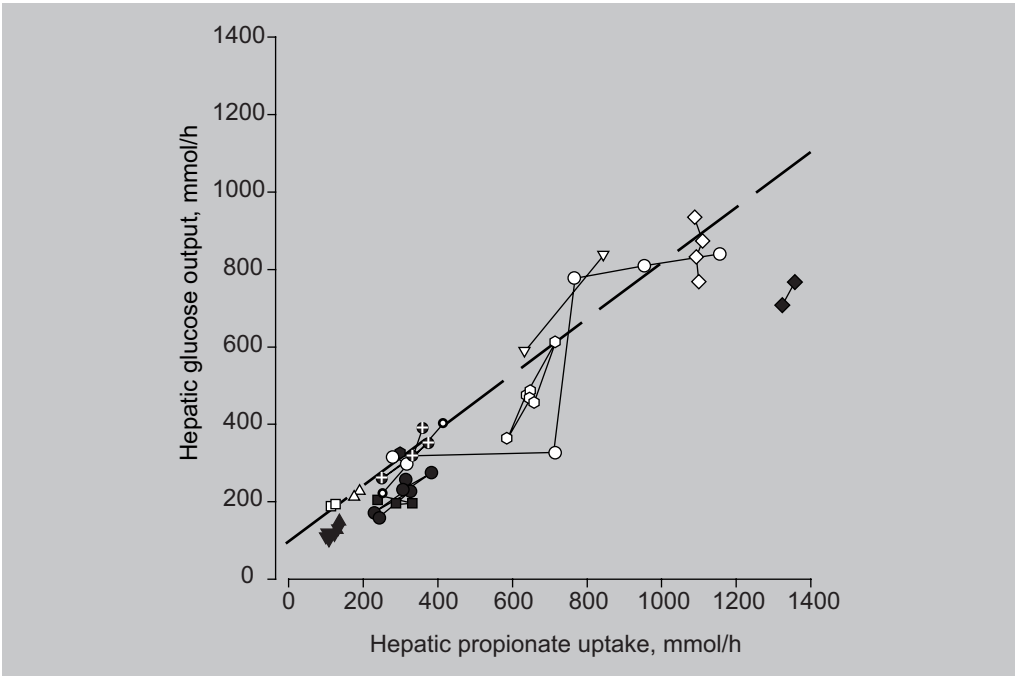

Figure 2. Hepatic propionate uptake and hepatic glucose output in cattle. Overall relationship between hepatic propionate uptake and glucose output: net hepatic glucose output (mmol/h) = 0.72 (±0.08) x net hepatic propionate uptake + 90 (±41) mmol/h. Data from: Eisemann et al.*, 1996 (●), Reynolds* et al.*, 2003 (○), Reynolds* et al.*, 1994/Reynolds and Tyrrell, 1991 (■), Reynolds* et al.*, 1992 (□), Harmon* et al.*, 1991b (▲), Harmon* et al.*, 1993 (△), Krehbiel* et al.*, 1992 (▼), Reynolds* et al.*, 1988a/Reynolds* et al.*, 1988b(▽), Casse* et al.*, 1994(◆), Benson* et al.*, 2002; Benson* et al.*, 2001 (◇), closed hexagon (Baird* et al.*, 1980), De Visser* et al.*, 1997 (⬡), Lomax and Baird, 1983 (⭘), Lozano* et al.*, 2000 (⊕).*

independently of feed intake, there is a large variation in glucose production responses (Table 2). Glucose production increases when propionate is supplied, however, increased glucose production (on a carbon basis) accounts for only 38 ±19% of the propionate carbon infused. In consequence, it does not appear that there exists a simple relationship between fermentation pattern (i.e. ruminal propionate production) and hepatic glucose production at a given total energy intake. These aspects could be important in attempts to model metabolic effects resulting from consumption of different rations.

Butyrate

The liver appears to play less of a role in butyrate metabolism than in propionate metabolism. Thus, not only is hepatic extraction of butyrate lower compared with propionate, but the fact that only ~25% of the absorbed butyrate is released into the portal vein means that the liver metabolizes ~20% of the ruminal butyrate production compared with ~90% of the propionate. One could hypothesize that the primary reason for rumen epithelial metabolism of butyrate (or hindgut in

Table 2. Recovery (%) of an increased propionate supply [mmol carbon x $(BW^{0.75})^{-1}$ x d^{-1}] in increased glucose production in sheep, steers or dairy cows supplied with propionate in the feed, by intraruminal infusion or from buffered solutions in washed rumen experiments. Glucose production [mmol carbon x $(BW^{0.75})^{-1}$ x d^{-1}] was measured either from isotopic dilution of radio or stable isotope labeled glucose or by net hepatic glucose fluxes in multicatheterized animals.

Species	Site of infusion	Duration, d	Method	Propionate infusion	Recovery in glucose production, %	Total glucose production[1]	Reference
Sheep	Rumen	< 1	Isotopic dilution	409	8	222	Judson and Leng (1973)
Sheep	Rumen	8	Net flux	130 183	0 8	331 346	Majdoub *et al.* (2003)
Steers	Washed rumen	< 1	Net flux	211	6	263	Kristensen and Harmon (2004c)
Steers	Rumen	7	Isotopic dilution	40 79	-4 76	449 511	Seal and Parker (1994)
Steers	Feed	25	Isotopic dilution	28	217	463	Peiris *et al.* (1998)
Steers	Feed	28	Isotopic dilution	363	42	407	Veenhuizen *et al.* (1988)
Dairy cows	Washed rumen	< 1	Isotopic dilution	362	37	528	Kristensen *et al.* (2002)
Dairy cows	Feed	14	Isotopic dilution	158 317	28 8	677 657	Lemosquet *et al.* (2004)

[1]Total glucose production = glucose production during propionate infusion

non-ruminants) is to keep butyrate away from the liver, thereby avoiding competition between propionate and butyrate for acyl-CoA synthetases. By compartmentalizing their metabolisms in different tissues, substrate pools in both tissues would remain homogenous with limited substrate competition. To what extent this explanation can account for the different SCFA in ruminal epithelium and liver is not known, but it is striking that the liver's affinity for propionate is high, but relatively low for butyrate, and again high for valerate and other longer chain fatty acids (Kristensen and Harmon, 2004b).

Even in non-ketotic dairy cows, the liver releases more 3-hydroxybutyrate than can be accounted for by butyrate uptake. Although the ruminal epithelium metabolizes ~75% of the butyrate supply, it releases only ~50% of the 3-hydroxybutyrate produced in the splanchnic tissues (Casse *et al.*, 1994; Reynolds *et al.*, 1988a; Reynolds *et al.*, 2003). This value is obtained after correction for

PDV uptake of arterial 3-hydroxybutyrate (Kristensen *et al*., 2000c). The carbon sources for the 3-hydroxybutyrate released by the liver is probably, in addition to butyrate, acetoacetate taken up from the portal blood (Lomax and Baird, 1983) and lipid (Bell, 1980).

Valerate, caproate, and heptanoate

Valerate, caproate and heptanoate are all efficiently taken up by the liver with caproate the most efficiently extracted of the SCFA we have examined so far (Kristensen and Harmon, 2004b). In consequence, peripheral tissues are presented with very low concentrations of these acids. Caproate is strongly ketogenic whereas valerate and heptanoate are catabolized to propionyl-CoA and, therefore, these are less ketogenic compared with caproate.

Branched-chain SCFA

In dairy cows, hepatic extraction of branched-chain SCFA (0.56 to 0.71) is generally higher than butyrate (Reynolds *et al*., 2003). Increased hepatic extraction ratios with increasing portal supply of these acids were observed in steers under washed rumen conditions (Kristensen and Harmon, 2004c). The increased extraction ratio indicates that there is an element of allosteric regulation in the liver uptake of branched-chain SCFA. The CoA esters of different branched-chain SCFA are intermediates in animal catabolism of their respective amino acids from which they also originate in the rumen i.e. isobutyrate from valine, isovalerate from leucine, and 2-methylbutyrate from isoleucine (Bender, 1985), and it is assumed that the metabolism of the branched-chain SCFA follow the same pathways, i.e. that isovalerate is purely ketogenic and metabolized via HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) to acetyl-CoA and acetoacetate, that isobutyrate is metabolized to succinyl-CoA and that 2-methylbutyrate is metabolized into acetyl-CoA + propionyl-CoA.

Acyl-CoA synthetases in SCFA metabolism

Activation of SCFA by an acyl-CoA synthetase (also referred to as CoA ligase or thiokinase) is the first step in metabolism of all SCFA and of other fatty acids in the gut epithelium, liver, and peripheral tissues (Groot *et al*., 1976). The acyl-CoA synthetases are therefore believed to be key enzymes in a tissue's selectivity for metabolism of the different SCFA. The different affinities of the ruminal epithelium and liver for a SCFA (Figure 1), therefore are likely to be a direct consequence of the differences in the first step of their intracellular metabolism, i.e. their 'activation'. The SCFA are activated by formation of a thioester with CoA-SH and there exist a number of distinct acyl-CoA synthetases: acetyl-CoA-, propionyl-CoA-, butyryl-CoA-, medium-chain fatty acid-, long-chain fatty acid-CoA synthetases, and more.

Acetyl-CoA synthetase (EC 6.2.1.1) has a high affinity for acetate, and some affinity for propionate (Campagnari and Webster, 1963; Groot *et al*., 1976; Ricks and Cook, 1981b). However, it is noteworthy that the activity of this enzyme is low in the ruminal epithelium and liver of ruminants (Cook *et al*., 1969; Ash and Baird, 1973). These observations are in line with the limited role of the ruminal epithelium and the liver in metabolism of absorbed acetate.

The ruminant liver is relatively abundant in propionyl-CoA synthetase (EC 6.2.1.17) activity (Ash and Baird, 1973) and there exist a number of indications that propionyl-CoA synthetase is a distinct enzyme (Ricks and Cook, 1981c; Ricks and Cook, 1981a). Among the interesting features of this enzyme is that it is not present in ruminal epithelium. This is not the same as denying activation of propionate in ruminal epithelium, which obviously can occur (Weekes, 1974), but it has been shown that the propionyl-CoA synthetase activity in the liver is nearly insensitive to the presence of butyrate. By contrast, the activity of this enzyme in ruminal epithelium is almost completely inhibited by the presence of butyrate (Ash and Baird, 1973; Harmon *et al*., 1991a). As is the case with acetyl-CoA synthetase in rumen epithelium, the lack of propionyl-CoA synthetase activity is in agreement with *in vivo* observations of limited uptake of propionate by the ruminal epithelium. As stated above, butyrate inhibits hepatic propionate uptake *in vitro* as well as in short term *in vivo* experiments (Aiello *et al*., 1989; Kristensen and Harmon, 2004a), however, this may not be due to direct competitive interactions at the level of propionyl-CoA synthetase because this enzyme has a very low affinity for butyrate. Another, but still unresolved, possibility is that the same type of regulation observed for the branched-chain SCFA and for valerate activation by the liver also is important in the propionate/butyrate interaction. It is likely that propionate/butyrate interactions in the liver are important primarily when the supply changes over short periods of time. In long term *in vivo* experiments, hepatic propionate extraction is apparently not reduced in animals fed diets inducing high ruminal butyrate production compared with diets inducing low ruminal butyrate production (Berthelot *et al*., 2002).

As described above one of the most striking features of rumen epithelial metabolism is a high affinity and high capacity for metabolism of butyrate. This feature is reflected in the butyryl-CoA synthetase activity of the epithelium (Ash and Baird, 1973). Butyrate has an insignificant effect on propionate activation in the liver and propionate has no effect on butyrate activation in the ruminal epithelium, but decreased the butyrate activation in the liver (Ash and Baird, 1973). A distinct butyryl-CoA synthetase (EC 6.2.1.2) was first purified from bovine heart mitochondria and this enzyme showed a high affinity for valerate and caproate (Webster *et al*., 1965). In ruminants, butyrate affinity is also found in xenobiotic/medium-chain fatty acid-CoA synthetases. These acyl-CoA synthetases activate a broad spectrum of straight chain fatty acids: butyrate, longer SCFA, branched chain fatty acids, and a number of xenobiotic (of foreign origin) carboxylic acids; others include benzoate and phenyl acetate (Aas, 1971; Vessy *et al*., 1999). Indirect evidence from PDV flux studies indicates cross specificity for valerate activation, which agrees with both types of butyrate activating systems. So far, no specific information seems to be available on the interaction of SCFA activation with longer chain fatty acids or xenobiotic compounds absorbed from the rumen (Cremin *et al*., 1995); however, the fact that the isolated ruminal epithelium or isolated rumen epithelial cells are able to use a wide range of fatty acids from SCFA to palmitate indicates the presence of some activity for activating medium- as well as long chain fatty acids by the epithelium (Jesse *et al*., 1992; Hird *et al*., 1966). In agreement with these *in vitro* observations it was found in steers that approximately 50% of the caproate and heptanoate absorbed from the washed rumen was recovered in the portal vein indicating activity for MCFA activation (Kristensen and Harmon, 2004b).

Perspectives

Nutrition has a strong impact on most aspects of animal husbandry and developments within nutritional sciences are therefore of fundamental importance to almost all efforts to improve animal husbandry no matter if the primary objective is animal welfare, product quality, environmental impact of the production, economical efficiency, or a combination of objectives. It is generally accepted that traditional, empirically based feed evaluation systems have difficulties solving today's problems in animal agriculture because of the complex interactions between economic and biological aspects of the production systems. However, even though this basically has been recognized for decades, we are still struggling to add up all the specific metabolites supplied to ruminant metabolism from intestinal fermentation and digestion. The long-standing uncertainty of the availability of SCFA is just one example of the gap between digestion and metabolism that has persisted to exist in our knowledge on quantitative nutritional physiology of the ruminant. Also, our understanding of the relationships between intestinal availability and tissue availability for the nitrogenous compounds has changed considerably in recent years (Reynolds, 2002). The recent work in these areas has taken us an important step forward in terms of accounting for nutrient inputs to ruminant metabolism and shown that the multicatheterized animal model in combination with isotope techniques has a great potential for further exploration of farm animal biology and for providing the basic knowledge necessary for progress in development of nutrient-based feed evaluation systems.

Acknowledgements

Financial support from the University of Kentucky Agricultural Experimental Station and the Danish Institute of Agricultural Sciences made preparation of the present paper possible and is gratefully appreciated.

References

Aas, M. (1971). Organ and subcellular distribution of fatty acid activating enzymes in the rat. Biochimica et Biophysica Acta, 231, 32-47.

Aiello, R.J., L.E. Armentano, S.J. Bertics & A.T. Murphy (1989). Volatile fatty acid uptake and propionate metabolism in ruminant hepatocytes. Journal of Dairy Science, 72, 942-949.

Ash, R. & G.D. Baird (1973). Activation of volatile fatty acids in bovine liver and rumen epithelium. Evidence for control by autoregulation. Biochemical Journal, 136, 311-319.

Baird, G.D., M.A. Lomax, H.W. Symonds & S.R. Shaw (1980). Net hepatic and splanchnic metabolism of lactate, pyruvate and propionate in dairy cows in vivo in relation to lactation and nutrient supply. Biochemical Journal, 186, 47-57.

Bell, A.W. (1980). Lipid metabolism in liver and selected tissues and in the whole body of ruminant animals. Progress in Lipid Research, 18, 117-164.

Bender, D. (1985). Amino Acid Metabolism (2 ed.). Chichester, GB: John Wiley & Sons.

Benson, J.A., C.K. Reynolds, P.C. Aikman, B. Lupoli & D.E. Beever (2002). Effects of abomasal vegetable oil infusion on splanchnic nutrient metabolism in lactating dairy cows. Journal of Dairy Science, 85, 1804-1814.

Benson, J.A., C.K. Reynolds, D.J. Humphries, S.M. Rutter & D.E. Beever (2001). Effects of abomasal infusion of long-chain fatty acids on intake, feeding behavior and milk production in dairy cows. Journal of Dairy Science, 84, 1182-1191.

Bergman, E.N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological Reviews, 70, 567-589.

Bergman, E.N. & J.E. Wolff (1971). Metabolism of volatile fatty acids by liver and portal-drained viscera in sheep. American Journal of Physiology, 221, 586-592.

Berthelot, V., S.G. Pierzynowski, D. Sauvant & N.B. Kristensen (2002). Hepatic metabolism of propionate and methylmalonate in growing lambs. Livestock Production Science, 74, 33-43.

Britton, R. & C. Krehbiel (1993). Nutrient metabolism by gut tissues. Journal of Dairy Science, 76, 2125-2131.

Bugaut, M. (1987). Occurrence, absorption and metabolism of short chain fatty acids in the digestive tract of mammals. Comparative Biochemistry and Physiology, 86B, 439-472.

Campagnari, F. & L.T.Jr. Webster (1963). Purification and properties of acetyl coenzyme A synthetase from bovine heart mitochondria. The Journal of Biological Chemistry, 238, 1628-1633.

Casse, E.A., H. Rulquin & G.B. Huntington (1994). Effect of mesenteric vein infusion of propionate on splanchnic metabolism in primiparous holstein cows. Journal of Dairy Science, 77, 3296-3303.

Cook, R.M., S.-C.C. Liu & S. Quraishi (1969). Utilization of volatile fatty acids in ruminants. III. Comparison of mitochondrial acyl coenzyme A synthetase activity and substrate specificity in different tissues. Biochemistry, 8, 2966-2969.

Cremin, J.D.Jr., K.R. McLeod, D.L. Harmon, A.L. Goetsch, L.D. Bourquin & G.C.Jr. Fahey (1995). Portal and hepatic fluxes in sheep and concentrations in cattle ruminal fluid of 3-(4-hydrophenyl)propionic, benzoic, 3-phenylpropionic, trans-cinnamic acids. Journal of Animal Science, 73, 1766-1775.

Crichlow, E.C. (1988). Ruminal lactic acidosis: forestomach epithelial receptor activation by undissociated volatile fatty acids and rumen fluids collected during loss of reticuloruminal motility. Research in Veterinary Science, 45, 364-368.

Danfær, A. (1994). Nutrient metabolism and utilization in the liver. Livestock Production Science, 39, 115-127.

Danfær, A., V. Tetens & N. Agergaard (1995). Review and an experimental study on the physiological and quantitative aspects of gluconeogenesis in lactating ruminants. Comparative Biochemistry and Physiology, 111B, 201-210.

De Visser, H., H. Valk, A. Klop, J. Van der Meulen, J.G.M. Bakker & G.B. Huntington (1997). Nutrient fluxes in splanchnic tissue of dairy cows: Influence of grass quality. Journal of Dairy Science, 80, 1666-1673.

Eisemann, J.H., G.B. Huntington & D.R. Catherman (1996). Patterns of nutrient interchange and oxygen use among portal-drained viscera, liver, and hindquarters of beef steers from 235 to 525 kg body weight. Journal of Animal Science, 74, 1812-1831.

Gálfi, P., S. Neogrády & T. Sakata (1991). Effects of volatile fatty acids on the epithelial cell proliferation of the digestive tract and its hormonal mediation. In Tsuda, T., Y. Sasaki & R. Kawashima (eds.), Physiological aspects of digestion and metabolism in ruminants. San Diego, California: Academic Press Inc. 49-59.

Groot, P.H.E., H.R. Scholte & W.C. Hülsmann (1976). Fatty acid activation: specificity, localization, and function. Adv.Lipid Res., 14, 75-126.

Gross, K.L., D.L. Harmon & T.B. Avery (1990). Portal-drained visceral flux of nutrients in lambs fed alfalfa or maintained by total intragastric infusion. Journal of Animal Science, 68, 214-221.

Harmon, D.L., K.L. Gross, C.R. Krehbiel, K.K. Kreikemeier, M.L. Bauer & R.A. Britton (1991a). Influence of dietary forage and energy intake on metabolism and acyl-coa synthetase activity in bovine ruminal epithelial tissue. Journal of Animal Science, 69, 4117-4127.

Harmon, D.L., K.L. Gross, K.K. Kreikemeier, K.P. Coffey, T.B. Avery & J. Klindt (1991b). Effects of feeding endophyte-infected fescue hay on portal and hepatic nutrient flux in steers. Journal of Animal Science, 69, 1223-1231.

Harmon, D.L., K.K. Kreikemeier & K.L. Gross (1993). Influence of addition of monensin to an alfalfa hay diet on net portal and hepatic nutrient flux in steers. Journal of Animal Science, 71, 218-225.

Hird, F.J.R., R.B. Jackson & M.J. Weidemann (1966). Transport and metabolism of fatty acids by isolated rumen epithelium. Biochemical Journal, 98, 394-400.

Huntington, G.B. (1990). Energy metabolism in the digestive tract and liver of cattle: influence of physiological state and nutrition. Reproduction Nutrition Development, 30, 35-47.

Huntington, G.B., C.K. Reynolds & B.H. Stroud (1989). Techniques for measuring blood flow in splanchnic tissues of cattle. Journal of Dairy Science, 72, 1583-1595.

Jesse, B.W., R.K. Solomon & R.L. VI Baldwin (1992). Palmitate metabolism by isolated sheep rumen epithelial cells. Journal of Animal Science, 70, 2235-2242.

Judson, G.J. & R.A. Leng (1973). Studies on the control of gluconeogenesis in sheep: effect of propionate, casein and butyrate infusions. British Journal of Nutrition, 29, 175-195.

Knowles, S.E., I.G. Jarrett, O.H. Filsell & F.J. Ballard (1974). Production and utilization of acetate in mammals. Biochemical Journal, 142, 401-411.

Krehbiel, C.R., D.L. Harmon & J.E. Schnieder (1992). Effect of increasing ruminal butyrate on portal and hepatic nutrient flux in steers. Journal of Animal Science, 70, 904-914.

Kristensen, N.B. & A. Danfær (2001). The relationship between gastrointestinal production and portal absorption of short-chain fatty acids in ruminants. In Chwalibog, A. & K. Jakobsen (eds.), Energy Metabolism in Animals. Proceedings of the 15th Symposium on Energy Metabolism in Animals Wageningen: Wageningen Pers. 277-280.

Kristensen, N.B., A. Danfær & N. Agergaard (1998). Absorption and metabolism of short-chain fatty acids in ruminants. Archives of Animal Nutrition, 51, 165-175.

Kristensen, N.B., A. Danfær, B.A. Røjen, B.-M.L. Raun, M.R. Weisbjerg & T. Hvelplund (2002). Metabolism of propionate and 1,2-propanediol absorbed from the washed reticulorumen of lactating cows. Journal of Animal Science, 80, 2168-2175.

Kristensen, N.B., A. Danfær, V. Tetens & N. Agergaard (1996). Portal recovery of intraruminally infused short-chain fatty acids in sheep. Acta Agriculturae Scandinavica Section A - Animal Science, 46, 26-38.

Kristensen, N.B., G. Gäbel, S.G. Pierzynowski & A. Danfær (2000a). Portal recovery of short-chain fatty acids infused into the temporarily-isolated and washed reticulo-rumen of sheep. British Journal of Nutrition, 84, 477-482.

Kristensen, N.B. & D.L. Harmon (2004a). Effect of increasing ruminal butyrate absorption on splanchnic metabolism of VFA absorbed from the washed reticulorumen of steers. Journal of Animal Science, 82, 3549-3559.

Kristensen, N.B. & D.L. Harmon (2004b). Effect of increasing ruminal valerate, caproate, and heptanoate on splanchnic metabolism of VFA absorbed from the washed reticulorumen of steers. Journal of Animal Science, 82 (suppl. 1), 272.

Kristensen, N.B. & D.L. Harmon (2004c). Splanchnic metabolism of VFA absorbed from the washed reticulorumen of steers. Journal of Animal Science, 82, 2033-2042.

Kristensen, N.B., S.G. Pierzynowski & A. Danfær (2000b). Net portal appearance of volatile fatty acids in sheep intraruminally infused with mixtures of acetate, propionate, isobutyrate, butyrate, and valerate. Journal of Animal Science, 78, 1372-1379.

Kristensen, N.B., S.G. Pierzynowski & A. Danfær (2000c). Portal-drained visceral metabolism of 3-hydroxybutyrate in sheep. Journal of Animal Science, 78, 2223-2228.

Le Bars, H., J. Lebrument, R. Nitescu & H. Simonnet (1954). Recherches sur la motricité du rumen chez les petits ruminants. IV. Action de l'injection intraveineuse d'acides gras à courte chaîne. Bulletin Academie. Veterinaire, France, 27, 53-67.

Lemosquet, S., S. Rigout, A. Bach, H. Rulquin & J.W. Blum (2004). Glucose metabolism in lactating cows in response to isoenergetic infusions of propionic acid or duodenal glucose. Journal of Dairy Science, 87, 1767-1777.

Leng, R.A. & E.F. Annison (1963). Metabolism of acetate, propionate and butyrate by sheep-liver slices. Biochemical Journal, 86, 319-327.

Lomax, M.A. & G.D. Baird (1983). Blood flow and nutrient exchange across the liver and gut of the dairy cow. British Journal of Nutrition, 49, 481-496.

Lozano, O., C.B. Theurer, A. Alio, J.T. Huber, A. Delgado-Elorduy, P. Cuneo, D. Deyoung, M. Sadik & R.S. Swingle (2000). Net absorption and hepatic metabolism of glucose, L-lactate, and volatile fatty acids by steers fed diets containing sorghum grain processed as dry-rolled or steam-flaked at different densities. Journal of Animal Science, 78, 1364-1371.

Majdoub, L., M. Vermorel & I. Ortiques-Marty (2003). Intraruminal propionate supplementation modifies hindlimb energy metabolism without changing the splanchnic release of glucose in growing lambs. British Journal of Nutrition, 89, 39-50.

Manns, J.G. & J.M. Boda (1967). Insulin release by acetate, propionate, butyrate, and glucose in lambs and adult sheep. American Journal of Physiology, 212, 747-755.

Ørskov, E.R., N.A. MacLeod, R.N.B. Kay & P.C. Gregory (1984). Method and validation of intragastric nutrition. Canadian Journal of Animal Science, 64 (Suppl), 138-139.

Peiris, H., R. Elliott & B.W. Norton (1998). Supplementary grain and sodium propionate increase the liveweight gain and glucose entry rates of steers given molasses diets. Journal of Agricultural Science, Cambridge, 130, 205-211.

Pennington, R.J. (1952). The metabolism of short-chain fatty acids in the sheep 1. Fatty acid utilization and ketone body production by rumen epithelium and other tissues. Biochemical Journal, 51, 251-258.

Pethick, D.W. & D.B. Lindsay (1982). Acetate metabolism in lactating sheep. British Journal of Nutrition, 48, 319-328.

Prasad, K.N. & P. Sinha (1976). Effect of sodium butyrate on mammalian cells in culture: A review. In Vitro, 12, 125-132.

Rémond, D., I. Ortiques & J.P. Jouany (1995). Energy substrates for the rumen epithelium. Proceedings of the Nutrition Society, 54, 95-105.

Reynolds, C.K. (1995). Quantitative aspects of liver metabolism in ruminants. In Engelhardt, W.v., S. Leonhard-Marek, G. Breves & D. Giesecke (eds.), Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction Stuttgart, Germany: Ferdinand Enke Verlag. 351-371.

Reynolds, C.K. (2002). Economics of visceral energy metabolism in ruminants: Toll keeping or internal revenue service? Journal of Animal Science, 80 (Suppl. 2), E74-E84.

Reynolds, C.K., P.C. Aikman, B. Lupoli, D.J. Humphries & D.E. Beever (2003). Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. Journal of Dairy Science, 86, 1201-1217.

Reynolds, C.K., D.L. Harmon, R.L. Prior & H.F. Tyrrell (1994). Effects of mesenteric vein l-alanine infusion on liver metabolism of organic acids by beef heifers fed diets differing in forage:concentrate ratio. Journal of Animal Science, 72, 3196-3206.

Reynolds, C.K. & G.B. Huntington (1988). Partition of portal-drained visceral net flux in beef steers. 2. Net flux of volatile fatty acids, D-β-hydroxybutyrate and L-lactate across stomach and post-stomach tissues. British Journal of Nutrition, 60, 553-562.

Reynolds, C.K., G.B. Huntington, H.F. Tyrrell & P.J. Reynolds (1988a). Net metabolism of volatile fatty acids, D-β-hydroxybutyrate, nonesterified fatty acids, and blood gasses by portal-drained viscera and liver of lactating holstein cows. Journal of Dairy Science, 71, 2395-2405.

Reynolds, C.K., G.B. Huntington, H.F. Tyrrell & P.J. Reynolds (1988b). Net portal-drained visceral and hepatic metabolism of glucose, L-lactate and nitrogenous compounds in lactating holstein cows. Journal of Dairy Science, 71, 1803-1812.

Reynolds, C.K. & H.F. Tyrrell (1991). Effects of mesenteric vein L-alanine infusion on liver metabolism in beef heifers fed on diets differing in forage:concentrate ratio. British Journal of Nutrition, 66, 437-450.

Reynolds, C.K., H.F. Tyrrell & L.E. Armentano (1992). Effects of mesenteric vein n-butyrate infusion on liver metabolism by beef steers. Journal of Animal Science, 70, 2250-2261.

Ricks, C.A. & R.M. Cook (1981a). Partial purification of enzymes of bovine kidney mitochondria activating volatile fatty acids. Journal of Dairy Science, 64, 2344-2349.

Ricks, C.A. & R.M. Cook (1981b). Regulation of volatile fatty acid uptake by mitochondrial acyl CoA synthetases of bovine heart. Journal of Dairy Science, 64, 2336-2343.

Ricks, C.A. & R.M. Cook (1981c). Regulation of volatile fatty acid uptake by mitochondrial acyl CoA synthetases of bovine liver. Journal of Dairy Science, 64, 2324-2335.

Sander, E.G., R.G. Warner, H.N. Harrison & J.K. Loosli (1959). The stimulatory effect of sodium butyrate and sodium propionate on the development of rumen mucosa in the young calf. Journal of Dairy Science, 42, 1600-1605.

Seal, C.J. & D.S. Parker (1994). Effect of intraruminal propionic acid infusion on metabolism of mesenteric- and portal-drained viscera in growing steers fed a forage diet: I. Volatile fatty acids glucose and lactate. Journal of Animal Science, 72, 1325-1334.

Seal, C.J. & D.S. Parker (2000). Influence of gastrointestinal metabolism on substrate supply to the liver. In Cronjé, P.B. (ed.), Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction Oxon: CABI Publishing. 131-148.

Seal, C.J. & C.K. Reynolds (1993). Nutritional implications of gastrointestinal and liver metabolism in ruminants. Nutrition Research Reviews, 6, 185-208.

Sehested, J., L. Diernæs, P.D. Møller & E. Skadhauge (1999). Ruminal transport and metabolism of short-chain fatty acids (SCFA) in vitro: effect of SCFA chain length and pH. Comparative Biochemistry and Physiology, 123A, 359-368.

Stangassinger, M. & D. Giesecke (1979). Quantitative measurement of gluconeogenesis from isobutyrate in sheep. Archives Internationales de Physiologie et de Biochimie, 87, 265-274.

Sutton, J.D. (1985). Digestion and absorption of energy substrates in the lactating cow. Journal of Dairy Science, 68, 3376-3393.

Sutton, J.D., M.S. Dhanoa, S.V. Morant, J. France, D.J. Napper & E. Schuller (2003). Rates of production of acetate, propionate, and butyrate in the rumen of lactating dairy cows given normal and low-roughage diets. Journal of Dairy Science, 86, 3620-3633.

Sutton, J.D. & S.V. Morant (1978). Measurement of the rate of volatile fatty acid production in the rumen. In Osburn, D.F., D.E. Beever & D.J. Thomson (eds.), Ruminant Digestion and Feed Evaluation London: Agricultural Research Council. 7.1-7.11.

Topping, D.L. & P.M. Clifton (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiological Reviews, 81, 1031-1064.

Van den Bosch, H., R.B.H. Schutgens, R.J.A. Wanders & J.M. Tager (1992). Biochemistry of peroxisomes. Annual Review of Biochemistry, 61, 157-197.

Veenhuizen, J.J., R.W. Russell & J.W. Young (1988). Kinetics of metabolism of glucose, propionate and CO_2 in steers as affected by injecting phlorizin and feeding propionate. Journal of Nutrition, 118, 1366-1375.

Vessy, D.A., M. Kelly & R.S. Warren (1999). Characterization of the CoA ligases of human liver mitochondria catalyzing the activation of short- and medium-chain fatty acids and xenobiotic carboxylic acids. Biochimica et Biophysica Acta, 1428, 455-462.

Webster, L.T.Jr., L.D. Gerowin & L. Rakita (1965). Purification and characteristics of a butyryl coenzyme A synthetase from bovine heart mitochondria. The Journal of Biological Chemistry, 240, 29-33.

Weekes, T.E.C. (1974). The in vitro metabolism of propionate and glucose by the rumen epithelium. Comparative Biochemistry and Physiology, 49B, 393-406.

Weekes, T.E.C. & A.J.F. Webster (1975). Metabolism of propionate in the tissues of the sheep gut. British Journal of Nutrition, 33, 425-438.

Weigand, E., J.W. Young & A.D. McGilliard (1972). Extent of propionate metabolism during absorption from the bovine ruminoreticulum. Biochemical Journal, 126, 201-209.

Weigand, E., J.W. Young & A.D. McGilliard (1975). Volatile fatty acid metabolism by rumen mucosa from cattle fed hay or grain. Journal of Dairy Science, 58, 1294-1300.

Young, J.W., S.L. Thorp & H.Z. De Lumen (1969). Activity of selected gluconeogenic and lipogenic enzymes in bovine rumen mucosa, liver and adipose tissue. Biochemical Journal, 114, 83-88.

Part IV: Lactation and reproduction physiology

Patterns and putative regulatory mechanisms of high-affinity glutamate transporter expression by ruminants

J.C. Matthews and G.L. Sipe
Department of Animal Science, University of Kentucky, Lexington 40546-0215, USA

Abstract

High-affinity, highly concentrative, L-glutamate uptake is integral to support nitrogen and carbon homeostasis of many cell-types and tissue-level metabolic "cycles". Given the physiological costs associated with forestomach fermentation (high ammonia loads and/or low dietary-derived carbohydrates), knowledge about how system X^-_{AG} transport capacity is achieved and regulated is of particular interest to ruminant physiologists. Accordingly, this review discusses glutamate transport activities (systems); system X^-_{AG} transport proteins; the importance of system X^-_{AG} transport capacity in support of liver, white adipose, and striated muscle tissue function; identified and putative regulatory mechanisms that control system X^-_{AG} transporter expression and function, including whole-body and tissue regulation, transcriptional regulation of GLT-1 expression, coordinated expression and function relationships between GLT-1 and glutamine synthetase, posttranscriptional regulation of EAAC1 functional capacity; and modulation of cattle carcass quality and expression of system X^-_{AG} transporters and glutamine synthetase by chlortetracycline, a compositional-gain altering "antibiotic." Research that has characterized the patterns of system X^-_{AG} transporter expression in sheep and/or cattle, and alteration of basal patterns to support altered metabolic demands in response to growth, physiological development under a typical commercial regimen, and alterations to finished cattle in response to sub-therapeutic feeding of chlortetracycline are presented and the ramifications discussed.

Keywords: gene expression, SLC1A1, SLC1A2, NF-*k*B, beta-catenin

Introduction

Glutamate (for convenience, the "L" form of amino acids is implied, unless indicated otherwise) is extensively utilized to support whole-animal hydrocarbon and nitrogen metabolism (Heitmann and Bergman, 1981; Wu, 1998; Nissim, 1999). The predominant metabolic role/fate of glutamate is tissue-specific. Glutamate can serve as a source of oxidizable fuel, precursor for amino acid synthesis, carbon receptor for alpha-amino- and ammonia-nitrogen derived from the catabolism of other amino acids, or substrate for de novo protein synthesis. For example, in the intestinal epithelia, both dietary and enteric glutamate serve as important sources of carbons for oxidation-derived metabolic energy and endogenous synthesis of alanine and other amino acids (Wu, 1998). In the liver, glutamate serves as precursor for glutamine (which is exported to blood), gluconeogenic carbons, and incorporation into de novo protein synthesis (Haussinger and Gerok, 1983). In kidney tissue, plasma and kidney-synthesized glutamate acts to regulate ammoniagenesis, serves as a precursor for incorporation into glutathione and extracellular matrix protein, and serves as a source of carbons for mitochondrial oxidation-derived energy (Welbourne and Matthews, 1999). In placental tissue, both fetal- (primarily) and maternal-derived glutamate serve as sources of

oxidizable fuel. Consequently, more maternal glucose is available for fetal use, while neurotoxic levels of glutamate are removed from the fetal circulation (Moores *et al*., 1994; Vaughn *et al*., 1995). In skeletal muscle (Biolo *et al*., 1995; Vesli *et al*., 2002) and subcutaneous fat (Kowalski and Watford, 1994), the production of glutamine from plasma glutamate is thought to constitute an important route for whole-body ammonia recovery and detoxification.

For ruminants, glutamate appears to be an especially important precursor of metabolic energy, serving as source of respiratory fuel and gluconeogenic carbons (Fahey and Berger, 1988). For example, in forage-fed lactating dairy cattle, about 43% of whole-animal glutamate is oxidized to CO_2, which is, relatively, slightly greater than that observed for aspartate, alanine and glutamine (about 28 to 35%) and approximates that for acetate, propionate, and butyrate (Black *et al*., 1990). Glutamate also may play an especially important role in N cycling in ruminants, given the large amount of NH_3 produced from microbial fermentation.

In terms of glutamate metabolism by individual ruminant tissues, glutamate oxidation by sheep small intestinal mucosa is extensive as only about 4% of gut-infused [^{14}C]glutamate appears in the portal blood as [^{14}C]-labeled glutamate and/or glutamine (Tagari and Bergman, 1978) and about 65 to 75% of enterally-derived glutamate is oxidized to CO_2 in a single pass. In contrast, gluconeogenesis by the liver accounts for about 25% of total glutamate turnover in sheep, constituting metabolically significant, if relatively small (4 to 6.2%), contribution to total plasma glucose (Heitmann *et al*., 1973; Heitmann and Bergman, 1981). In terms of whole-animal reserve gluconeogenic capacity, glutamate-derived gluconeogenesis by the kidney is important, accounting for 33 to 50% of glutamate-derived glucose in fasted and starved sheep, respectively (Heitmann and Bergman, 1981).

Despite differences in the metabolic fate of glutamate in tissues, most tissues need the ability to concentrate glutamate, as tissue glutamate concentrations typically exceed extracellular concentrations and extracellular glutamate concentration modulates glutaminase-, glutamine synthetase-, and alanine aminotransferase-dependent metabolic pathways (Welbourne and Nissim, 2001).

Because of the extensive utilization of dietary glutamate by the intestine (Tagari and Bergman, 1978; Reeds *et al*., 1996; Wu, 1998), the principle source of circulating glutamate is the liver. More specifically, under normal physiological conditions, glutamate produced from the deamination of glutamine by liver-type glutaminase, and subsequently exported into sinusoidal blood by periportal vein hepatocytes, is thought to be the principle source of glutamate for peripheral issue use. In contrast to this net export of glutamate by the liver, net users of glutamate include the lung, subcutaneous adipose, skeletal muscle, and intestine, whereas the brain and kidney generate high fluxes of glutamate but without any net utilization or production (Hediger and Welbourne, 1999; Patterson *et al*., 2002). It is of importance to note that tissues which are net users of glutamate also are either net exporters of glutamine (lung, adipose, skeletal muscle) and/or alanine (skeletal muscle, intestine). This understanding, plus the observations that the liver, brain, and kidney possess well-defined glutamate/glutamine cycles (Nissim, 1999), and that a decreased glutamate exchange capacity appears responsible for elevated plasma glutamate levels of patients suffering from age- and disease-induced cachexia of skeletal muscle (Hack *et al*., 1996), emphasize the

importance of transport capacity to normal peripheral tissue metabolism of glutamate (Bronson, 2000; van Milgen, 2002).

Despite the importance of glutamate to nitrogen and energy metabolism, and the understanding that liver plasma membrane capacity limits glutamate metabolism (Low *et al*., 1994; Nissim, 1999; Gegelashvili *et al*., 2001), very little is known about which glutamate transporters are expressed outside of the central nervous system. Furthermore, even less is understood about how the expression and functional capacity of glutamate transporters are regulated, especially in ruminants. Accordingly, the subsequent sections of this review focus on the function, expression, and regulation of system X^-_{AG} transporters in support of L-glutamate metabolism. However, the transport of D- and L-aspartate is equally well mediated by these high-affinity, high capacity transporters. Consequently, the reader is advised that factors that affect the expression and function of system X^-_{AG} "glutamate" transport capacity, ostensibly will affect the capacity for D- and L-aspartate transport and metabolism in an analogous manner.

Glutamate transport systems

In mammals, mediated absorption of anionic amino acids occurs by Na^+-dependent and Na^+-independent biochemical activities. High-affinity and concentrative uptake of anionic amino acids principally occurs by Na^+-dependent X^-_{AG} and X^-_A transport systems, which recognize only anionic amino acids and some of their analogues. System X^-_{AG} activity often is biochemically-defined as the high affinity (K_m = 1-50 μM), Na^+-dependent transport of L-glutamate or L-aspartate that is inhibitable by D-aspartate. System X^-_{AG} mediates the uniport uptake of Na^+ with L-glutamate, D-aspartate, or L-aspartate. In contrast to system X^-_{AG} activity, system X^-_A does not transport glutamate or its analogues (as reviewed by Matsuo *et al*., 2002). In addition, systems ASC and B^o also transport L-glutamate and L-aspartate, but only in acidic (pH < 5.5) environments. Na^+-independent uptake of anionic amino acids is mediated by system x^-_c or x^-_G. System x^-_C transport is an obligate exchange activity that typically mediates the exchange of L-glutamate for L-cystine, but which also recognizes D-glutamate. In contrast, system x^-_G only transports L-glutamate. The limited knowledge about tissue-specific distribution patterns of these anionic amino acid transport activities in ruminant tissues has been reviewed elsewhere (Matthews, 2000). However, recent advances in the biochemical, molecular, and regulatory properties of system X^-_{AG} transporters are reported and discussed below, with particular focus on ruminant systems, which has been a major focus of our research efforts for the last several years.

System X^-_{AG} transport proteins

The five non-human system X^-_{AG} transporter orthologs (and genes) are known as GLAST1 (SLC1A3), GLT-1 (SLC1A2), EAAC1 (SLC1A1), EAAT4 (SLC1A6), and EAAT5 (SLC1A7), whereas the human orthologs are referred to as EAAT1-5 (excitatory amino acid transport), respectively. Functionally, the process for transport by all system X^-_{AG} transporter isotypes is thought to involve extracellular binding and translocation of one amino acid and three Na^+, with reorientation of the transporter to the extracellular face of the membrane being driven by the intracellular-to-extracellular counter-transport of one K^+. In addition, either one OH^- is

countertransported or one H^+ cotransported (Danbolt, 2001). EAAT4 and EAAT5 also have a large inward chloride ion flux associated with their function, which may aid in the re-establishment of membrane potential by influencing cellular chloride permeability, and has led them to be most accurately described as that of glutamate-gated chloride channels (Arriza *et al*., 1997). Although system X^-_{AG} transporters typically transport Na^+ and anionic amino acids from the extracellular fluid into the cytosol, they are capable of reverse transport when the typically high intracellular K^+ levels are too low (Zerangue and Kavanaugh, 1996; Levy *et al*., 1998).

The molecular characterization of the system X^-_{AG} transporters has allowed the development of molecular reagents (cDNAs, antibodies,etc.) to study their tissue distribution patterns and, consequently, knowledge of which system X^-_{AG} transport protein(s) supports individual cell and tissue function. In addition, these tremendous accomplishments have made possible the study of whole-body and molecular factors that regulate transporter expression and function. Consequently, our ability to understand the importance of glutamate transport capacity to whole-animal nutrient homeostasis continues to expand. These accomplishments are reviewed below.

Importance of system X^-_{AG} transport capacity in support of tissue function

Liver

A primary function of the mammalian liver is to coordinate whole-body energy and nitrogen metabolism. Hepatic transport and intermediary metabolism of glutamate are critical to these processes as glutamate is a central substrate for hepatic ureagenesis, gluconeogenesis, glutathione production, *de novo* protein synthesis, and nitrogen shuttling via glutamine (Meijer *et al*., 1990; Matthews, 2000; Watford, 2000). An important aspect of glutamate metabolism in the liver is the heterogeneity of glutamate metabolism. Specifically, glutamine, arriving from the peripheral tissues via the portal vein, is efficiently absorbed by periportal hepatocytes by the hepatic system N activity (Kilberg *et al*., 1980) and deaminated by glutaminase (liver-type) to release ammonia and glutamate. The released ammonia is incorporated into carbamoyl phosphate (by carbamoyl phosphate synthetase) for ureagenesis, whereas glutamate is available for export, conversion to alpha-ketoglutarate (as an anapleurotic reaction to replenish the citric acid content), used for gluconeogenesis, used for protein synthesis, or transported back into the sinusoids. Ultimately, the end use of glutamate is dictated by whole-body energy and/or ammonia levels and blood pH. Once released into the sinusoidal blood, glutamate is available for absorption by the "down-stream" pericentral zone heptocytes, and/or use by peripheral tissues, after exiting the liver in the hepatic vein. If absorbed by pericentral zone hepatocytes, glutamate and the "scavenged" sinusoidal ammonia (that which escapes incorporation into urea by periportal hepatocytes), can be incorporated into glutamine by glutamine synthetase.

The characteristic expression of glutaminase and carbomoylphosphate synthetase by periportal hepatocytes, and the concomitant expression of glutamine synthetase (GS) by pericentral hepatocytes, are not known to overlap, at least along the hepatic acinii of rats, pigs, and humans (Moorman *et al*., 1989, 1990; Wagenaar *et al*., 1994). Therefore, periportal and perivenous hepatocytes likely also differ in their needs for glutamate transport capacity. The knowledge that hepatocytes possess Na^+-dependent and/or Na^+-independent anionic amino acid transport came

from studies using isolated, non-polarized, rat hepatocytes (Gaseously *et al*., 1981; Gebhardt and Meckel, 1983). The understanding that perivenous hepatocytes are responsible for the majority of the net uptake of glutamate in the liver came from studies (Taylor and Rennie, 1987; Haussinger *et al*., 1989) with rats that used antegrade versus retrograde perfusion of dioxygensin to selectively kill periportal or pericentral hepatocytes, respectively. Subsequently, a histoautoradiographic study (Stoll *et al*., 1991) of perfused glutamate, aspartate, and alpha-ketoglutarate confirmed that pericentral hepatocytes (which synthesize glutamine) also possess the greatest, and almost exclusive, ability to accumulate glutamate. Although these studies clearly demonstrated the dominant ability of pericentral hepatocytes to accumulate glutamate, this research also indicated that periportal hepatocytes also absorb glutamate, most probably by system x^-_c (Burger *et al*., 1989; Haussinger *et al*., 1989).

In addition to its essential role in the intercellular glutamine cycle, an important feature of total hepatic glutamate metabolism to whole-body metabolism is that the liver is a net exporter of glutamate. Because the pericentral hepatocytes are thought to be responsible for more than half of the net portal export of glutamate from the liver (Haussinger *et al*., 1989), it follows that periportal hepatocytes likely provide the remaining net exported glutamate. Accordingly, pericentral and periportal hepotcyte populations quantitatively contribute about equally to provisioning of peripheral tissues with glutamate. However, given that in mature (adult-like) livers the pericentral hepatocytes constitute about 5 to 10% of the total hepatocyte population, the proportional capacity of pericentral hepatocytes to export glutamate appears to be about five times more than for periportal hepatocytes. The relatively lower glutamate export capacity of periportal hepatocytes may well reflect their relatively higher gluconeogenic and ureogenic metabolic capacities (Meijer *et al*., 1990; Kilberg and Haussinger, 1992). That is, the lesser proportional ability of periportal hepatocytes to export glutamate likely results from their greater fractional use of glutamate to support amino acid intermediary metabolism (Watford, 2000).

Hepatocytes are polarized cells, possessing basal (sinusoidal; blood-facing) and apical (canalicular; bile-facing) membranes. Therefore, it is important to know if glutamate transport capacities are localized in basal and/or canalicular membrane aspects of hepatocytes. Localization of transport activities in the basolateral membrane begets the ability to extract and excrete glutamate into the sinusoidal blood, whereas apically-localized activities allow flux across the canalicular membrane and into bile. Using isolated membrane vesicles, system X^-_{AG} activity has been demonstrated in canalicular-enriched fractions and a Na^+-independent activity in the sinusoidal membrane domain (Ballatori *et al*., 1986; Cariappa and Kilberg, 1992). In contrast, others have measured both system X^-_{AG} activity, and a Na^+-independent (exchanger) glutamate transport activity, in sinusoidal membranes of rat hepatocytes (Low *et al*., 1992). Furthermore, the system X^-_{AG} activity was upregulated in response to catabolic hormones (corticosteroid, glucagon), whereas the Na^+-independent, glutamate exchanger activity (system x^-_c-like) was insensitive to alteration of whole-body catabolic status. These results suggest that system X^-_{AG} activity on the sinusoidal membrane facilitates adaptation of hepatocytes to handle the increase in sinusoidal ammonia loads that accompany catabolic stress. That is, possession of an increased concentrative glutamate transport capacity would supply more glutamate to serve as an acceptor of ammonia and/or a source of gluconeogenic carbons through the activities of GS and glutamate dehydrogenase,

respectively. Unfortunately, response of canalicular membrane glutamate transport capacity to induction of whole-body catabolic states has not been evaluated.

In conclusion, much still remains to be determined about the zonal distribution and sub-membrane localization of glutamate transport activities in hepatocytes, and how this distribution of transport activities supports whole-body intermediary metabolism of glutamate. Recently, however, the expression of GLT-1 and EAAC1, but not GLAST1 or EAAT4, by the liver of mature sheep and cattle has been identified by Northern and immunoblot analysis of whole-liver homogenates and/or crude membrane vesicles (Howell *et al*., 2001). Subsequently, we have determined that this same pattern of differential system X^-_{AG} transporter isoform expression also occurs in young but fully-ruminating sheep (Howell *et al*., 2003) and throughout the development of growing cattle (preliminary data, Gissendanner *et al*., 2003a). In addition, in rats at least, GLT-1 is now known to be localized to pericentral hepatocytes (Cadoret *et al*., 2002). Furthermore, as detailed below, evidence is starting to accumulate that suggests that the expression of at least system X^-_{AG} (GLT-1) transporters and enzyme that uses glutamate (glutamine synthetase) is coordinated in liver tissue (Cadoret *et al*., 2002).

White adipose tissue

Compared to hepatocytes and intestinal epithelium, relatively little is known about the *in vivo* amino acid metabolism of adipose tissues. One reason for this relatively limited knowledge may be the inherent difficulties of catheterizing the small arterioles and venules of fat tissues, leading to inconsistent measurement of arterial-venous nutrient fluxes across fat tissues. However, the recent development and use of microdialysis sampling techniques has allowed the determination of differences between concentrations of extracellular and arterial nutrients to be determined, with some caveats (Summers *et al*., 1998; Rolinski *et al*., 2001; Lange *et al*., 2002).

In terms of basal (postabsorptive) adipocyte tissue metabolism, subcutaneous fat is a net user of glutamate and net producer of glutamine, as determined by arteriovenous analysis (Frayn *et al*., 1991). The inguinal fat pad/adipocyte tissue of fed rats also appears to be a net user of glutamate and ammonia, and a producer of glutamine but not of alanine (Kowalski and Watford, 1994). Accordingly, these two studies gave rise to the concept that adipose tissues of adequately nourished mammals absorb glutamate to synthesize glutamine and, therefore, contribute to whole-body nitrogen metabolism through the production of glutamine and removal of ammonia from the blood. In contrast, it has been observed that there is no net release of glutamine or uptake of ammonia by the inguinal pad, if rats are starved and re-fed (Kowalski *et al*., 1997). Therefore, this group concluded that the substrates for GS come from intracellular proteolysis, not plasma. If this interpretation is proven to be correct, then mitochondrial transport of glutamate (del Arco *et al*., 2002; Begum *et al*., 2002) may be the critical transport process to support glutamate metabolism by adipocytes. Alternatively, as seen for kidney proximal tubule epithelia, it may be that the extent of mitochondrial metabolism of glutamate by adipose tissue is dictated by the interaction/competition between plasma and mitochondrial glutamate transport activities (Welbourne *et al*., 2001).

Clearly, much remains to be determined about the metabolic pathways and physiological consequences of glutamate metabolism by fat tissue, especially when the normal post-absorptive state is perturbed. To this end, the influence of a protein-catabolic state (22-h fast) on protein metabolism in lean versus obese women was measured (Patterson *et al*., 2002). Although the subcutaneous abdominal adipose tissue of both treatment groups demonstrated net uptake of glutamate and production of glutamine, a net production of alanine also was measured. Whether this difference in the pattern of alanine production observed for postabsorptive versus fasted subjects results from differences in the types of fat tissue measured, or metabolic state, remains to be determined.

Collectively, the rather limited literature on glutamate metabolism by fat tissue indicates that at least various white-fat tissues are net users of glutamate and producers of glutamine and, perhaps, alanine. Accordingly, it seems reasonable to suggest that the relative contribution of fat-synthesized glutamine to whole-body nitrogen metabolism is strongly influenced by the relative activity of glutamate and glutamine transporters (Ritchie *et al*., 2001), GS, and alanine transaminase. However, a limitation of all arteriovenous measurements is that the metabolic activities and relative capacities of all the cell types that constitute a given tissue cannot be delineated. Accordingly, the extent to which the above studies actually represent adipocyte metabolic activities that are separate from glutamate metabolism by vasculature endothelial cells, stromal, or white cells (Patterson *et al*., 2002), also remains to be determined.

As for system X^-_{AG} transporters, we are ignorant of any published reports of plasma membrane glutamate transport activity or transporters by fat tissues. However, we have recently evaluated the potential expression of system X^-_{AG} transporters by fat tissues of developing cattle (preliminary data, Gissendanner *et al*., 2003b). EAAC1 mRNA and protein, and GLT-1 mRNA, are expressed by subcutaneous, perirenal, omental adipose tissues of suckling, weanling age, and backgrounding steers (30, 184, 284 days of age, respectively) and by subcutaneous, perirenal, omental, mesenchymal, and interstitial adipose tissues of finished (423 days of age) cattle. The relative content of EAAC1 protein increased with development in the three applicable fat tissues. GS content also increased from suckling to backgrounding physiological states, but decreased dramatically in finished steers. Although speculative, these results suggest that the younger cattle (which had yet to fatten) used the increased glutamate uptake capacity to support GS, as both GLT-1 and GS content was upregulated. In contrast, the adipose tissues of fattened cattle had a lesser capacity to synthesize glutamine as GS levels were decreased. Although more speculative yet, it is convenient to suggest that the increased glutamate capacity in adipose tissues of fattening cattle would be used to support fatty acid chain elongation and triacylglyceride synthesis. That is, to the extent that glutamate could be used for oxidative fuel and/or a source of acetoacetate carbons, glucose could be "spared" for NADPH and glycerol production, which are critical metabolites required for fatty acid chain elongation and incorporation into triacylglycerides, respectively.

Striated muscle tissues

The uptake of plasma glutamate, intracellular conversion of glutamate to glutamine, and subsequent export to the plasma of synthesized glutamine by skeletal muscle is critical to the

proper functioning of intestinal epithelia and the immune system (Newsholme and Parry-Billings, 1990; Hack *et al*., 1996). Besides diminishing the synthesis and export of glutamine, a reduction in the capacity for plasma glutamate uptake by skeletal muscle is thought to cause intracellular deficiencies of glutamate, glutamine, glutathione, and citric acid cycle intermediates, but increased carbon dioxide levels (Hack *et al*., 1996; Holm *et al*., 1997; Ushmorov *et al*., 1999).

Glutamate absorption by mammalian skeletal muscle initially was thought to occur by a Na^+-independent process, based on conclusions drawn from a rat hindlimb perfusion study (Hundal *et al*., 1989). Subsequently, and in accordance with that observed in the muscle of barnacles (Revest and Baker, 1988), the expression of both Na^+-dependent and -independent glutamate uptake activities was identified in skeletal muscle using primary cultures of rat myotubules (Low *et al*., 1994). The Na^+-dependent uptake of L-glutamate activity was inhibited by both D- and L-aspartate and displayed an affinity constant for L-glutamate of 0.7 mM. Therefore, although the profile of substrates that inhibit Na^+-dependent uptake of L-glutamate by myotubules is consistent with system X^-_{AG} transporters, the affinity is too low. As for cultured rat myotubules (Low *et al*., 1994), cultured myogenic C2C12 cells also possess a Na^+-coupled glutamate uptake capacity with an affinity constant of about 0.6 mM for L-glutamate (Frank *et al*., 2002). Importantly, the velocity of this activity could be stimulated one-fold in the presence of 2-chloro adenosine, by a process that was not mediated by glutamate receptors (Frank *et al*., 2000), and which resulted in a physiologically relevant increase of intracellular Ca^{2+} concentrations (Frank *et al*., 2002). This latter observation suggests that the uptake of glutamate by skeletal muscle may serve as a more immediate form of inter-tissue signaling, as compared to just an increased capacity for intermediary metabolism that elevated intracellular levels of glutamate. Furthermore, if it is shown that the rise of intracellular Ca^{2+} concentrations results from the act of glutamate transport per se, and not a generic alteration of intracellular Na^+, K^+, and/or Cl^- concentrations, and/or membrane potential, which would occur with the induction of any ion-coupled transport process, then an important understanding about the differences in supplying anaplerotic glutamate carbons as glutamine versus glutamate (Rennie *et al*., 2001) may have been gained. Unfortunately, the biochemical characterization of potential system X^-_{AG} transporter isoforms was insufficient to allow delineation of specific family members, whereas molecular analyses for the expression of putative glutamate transporters was not performed.

As just described, a discrepancy apparently exists between the biochemically and molecularly defined identities of Na^+-dependent transporters expressed by skeletal muscle tissue and myocytes. Unfortunately, specific information about the molecular identity of potential glutamate transporters expressed by muscle tissue is very limited. EAAC1 mRNA has been identified in skeletal muscle (Kanai and Hediger, 1992), thus confirming the possibility that EAAC1 could account for the system X^-_{AG} activity found in skeletal (Revest and Baker, 1988; Low *et al*., 1994; Frank *et al*., 2002) myocytes. Recently, we have identified the presence of both mRNA and protein for two system X^-_{AG} transporters (EAAC1 and GLT-1) in the longissimus dorsi muscle of cattle (preliminary data, Gissendanner *et al*., 2003b). From a developmental and/or aging perspective, an important observation was that the amount of both EAAC1 and GLT-1 expressed was inversely proportional to developmental age. That is, the content of both EAAC1 and GLT-1 in this skeletal muscle of suckling animals was greater than for weanling-age, which was greater than that for post-weaning (backgrounding) or finished cattle. This reduction in apparent glutamate transport

capacity in skeletal muscle may reflect normal physiological development. For example, there may be a diminished need for plasma glutamate if intracellular glutamate production capacity increased. Alternatively, reduced EAAC1 and GLT-1 content may reflect a loss of peripheral tissue sensitivity to insulin, as cattle adapt from a monogastric-like (suckling animal) to a fully ruminant (post-weaning/backgrounding and fattening) metabolism, and/or an ontogenetic (aging) effect. In humans, it has been well demonstrated that the capacity for skeletal muscle uptake of glutamate is decreased in both elderly and non-insulin-dependent diabetic mellitus individuals (Hack *et al*., 1996; Holm *et al*., 1997). However, evaluation of system X^-_{AG} transporter protein expression by human skeletal muscle and the putative sensitivity of transporter expression to loss of insulin stimulation are unknown to us.

Characterized and putative regulatory mechanisms of system X^-_{AG} transporter expression and function

Whole-body and tissue regulation

Information regarding substrate regulation of system X^-_{AG} transporter expression and activity is limited, especially with regard to whole-animal studies. With respect to substrate regulation of EAAC1 and GLT-1 expression, evidence exists that the content of GLT-1 and EAAC1 in brain tissue are differentially regulated by L-glutamate in brain tissue (Duan *et al*., 1999; Munir *et al*., 2000). In cell culture models, EAAC1 and/or GLT-1 expression by epithelial cells are sensitive to extracellular glutamate concentrations (Nicholson and McGivan, 1996; Novak *et al*., 2001). Even less is known about how the in vivo function of system X^-_{AG} transporters is regulated to support whole-body growth and maintenance. In eaac-$1^{-/-}$ mice, intestinal and kidney phenotypic development is normal, as are plasma amino acid levels (Peghini *et al*., 1997). However, the concentration of glutamate and aspartate in the urine of these animals is elevated 1,400- and 10-fold, respectively, as compared to wild-type mice. In contrast, in glt-1 -/- mice, urine glutamate and aspartate concentrations are unchanged but kidney glutamate and glutathione concentrations are markedly reduced (Tanaka and Welbourne, 2001).

Together, these gene knockout studies clearly indicate that at least two of the system X^-_{AG} transporters support different functions within the same tissue. We recently conducted a growth trial using sheep to determine (a) whether the ileal epithelium, hepatic, and/or renal tissue expression of GLT-1 and EAAC1 differed in lambs fed to gain versus maintain body weight and (b) whether a relationship existed between tissue content of D- and L-glutamate and EAAC1 or GLT-1 (Howell *et al*., 2003). A pertinent observation from this study was the increased expression of EAAC1 by ileal epithelial and GLT-1 by liver tissue membranes of growing versus non-growing lambs. Coincident with increased glutamate transporter content was an increased concentration of L-glutamate in ileal, and D-glutamate in hepatic, cellular membranes. That EAAC1 content was elevated in ileal epithelia that contained elevated L-glutamate (29.3 vs 43.7 nmol/mg), but GLT-1 was not, suggests that in vivo EAAC1 expression may have been stimulated by the presence of L-glutamate, whereas GLT-1 was not. Alternatively, the elevated glutamate levels could have resulted from elevated EAAC1 function, which in turn, was increased in response to some other cellular signal. By whatever physiological stimulus, this whole-animal study supports the

understanding that the dual expressions of EAAC1 and GLT-1 by small intestinal epithelia and the liver are independently regulated.

Obvious candidates for coordination of the differential expression of system X^-_{AG} transporters are the hormones. However, little is known regarding hormonal control of system X^-_{AG} transporter expression. Specifically, culture of C6 glioma cells with platelet-derived growth factor (PDGF) induces increased system X^-_{AG} activity and EAAC1 content in appearance in plasma membranes, consistent with glutamate supporting PDGF-stimulated proliferation responses (Sims *et al*., 2000). Research with transgenic mice (Matthews *et al*., 1999) indicates that placental expression of GLT-1 is stimulated by atypically high growth hormone levels and that IGF-II is required for GLT-1 and EAAC1 expression. Recent preliminary data from our lab (J. C. Matthews, unpublished data) indicates that expression of system X^-_{AG} activity by primary cultures of steer hepatocytes is stimulated by insulin. Common to both PDGF- and insulin-induced secondary messenger pathways is the dependence on tyrosine kinase and phosphatidylinositol-3 kinase (PI3K) activation. Therefore, given the elevated nutritional status of the growing vs non-growing lambs study (Howell *et al*., 2003), it is likely that both insulin/PDGF and L-glutamate levels were elevated in the ileal epithelium in growing lambs, and that a combination of these regulatory proteins factors resulted in the increased expression of EAAC1.

Transcriptional regulation of GLT-1 expression

The critical questions to understanding how the expression and/or functional capacity of a protein changes in response to a physiological state are: at what level, and by which mechanism, did the regulation occur? Collectively, the above data clearly indicate that regulation of system X^-_{AG} proteins is complex and likely involves transcriptional, post-transcriptional, and post-translational regulation, depending on the physiological status of a given cell, tissue, and/or organism.

Our research with the expression of system X^-_{AG} transporters by placenta, small intestine epithelia, liver, and kidney (Matthews *et al*., 1998, 1999; Howell *et al*., 2003; preliminary data, Gissendanner *et al*., 2003a) indicates that posttranscriptional regulation is an important form of regulating EAAC1, GLT-1, and EAAT4 because the presence or relative content of mRNA often is not mirrored by transporter protein content. Research from other groups also indicates that steady-state levels of glutamate transporter proteins do not always correlate with mRNA levels. For example, increased amounts of EAAC1 protein expression by amino acid-deprived Madin-Darby Bovine Kidney (MDBK) cells is accompanied by a decrease in EAAC1 mRNA levels (Plakidou-Dymock and McGivan, 1993).

In general, the expression and function of system X^-_{AG} transporters are regulated by growth factors, phosphorylation events, and interaction with transporter-binding proteins. In CNS tissues and cells, Na^+-dependent glutamate transporter subtypes appear sensitive to both transcriptional and post-translational regulatory mechanisms (Schlag *et al*., 1998; Schluter *et al*., 2002). In addition, several intracellular signaling pathways have been identified that are independent of de novo transporter synthesis (González *et al*., 2002). Several pathways have been identified (primarily

using glial and neuronal cell models) as being at least partially responsible for regulating the transcription of the GLT-1 gene (SLC1A2). These pathways are outlined in Figure 1.

In cortical astroglial cells, both GLT-1 mRNA and protein content are upregulated when the epidermal growth factor receptor (EGFR) pathway is activated. A number of EGFR ligands can induce this effect, including EGF and transforming growth factor alpha (TGFα) (Zelenaia *et al.*,

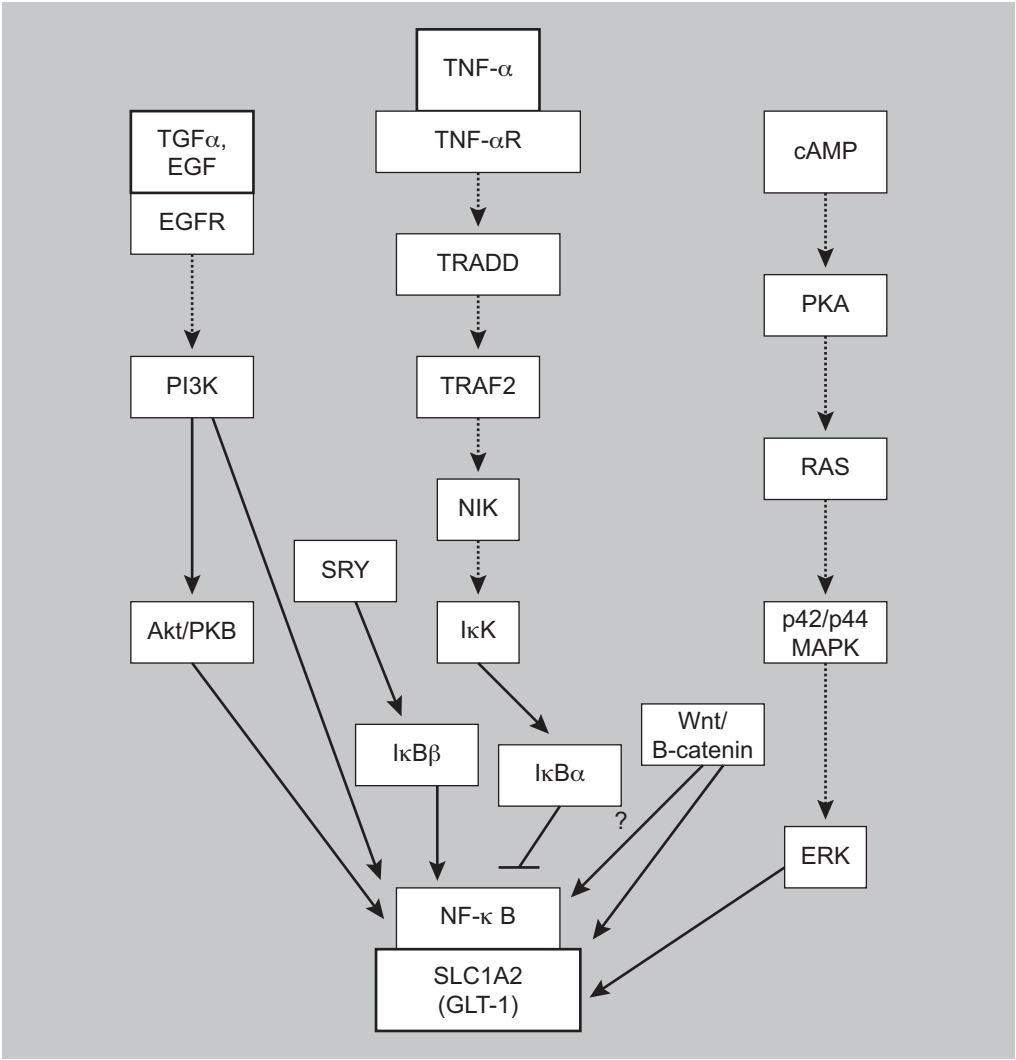

Figure 1. Working model for transcriptional control of SLC1A2 (gene for GLT-1). Pathway details and references are provided in the text. Both PI3K and IkBβ are able to stimulate (solid arrows) GLT-1 expression through independent but converging pathways. Separate activation of SLC1A2 by the Wnt/β-catenin and PKA pathways is indicated, whereas TNF-α inhibits (crossed solid lines) activation through activation of IκBα.

2000; Schluter *et al*., 2002; Figiel *et al*., 2003). Activation of the cyclic AMP (cAMP) pathway induces a large (up to 20-fold) increase in GLT-1 mRNA (Schlag *et al*., 1998). More specifically, alteration of GLT-1 expression is dependent on protein kinase A (PKA) activation (Robinson, 1999; Zelenaia *et al*., 2000). Because the onset of increased mRNA expression typically is not immediate (requiring more than several hours) it appears that cAMP-induction of GLT-1 expression occurs indirectly through protein kinase A (PKA)-induced expression of other transcription factors that bind SLC1A2 and/or increase the stability of GLT-1 mRNA.

Nuclear factor κ-B (NF-κB) is a sequence-specific transcriptional activation factor that was originally described for recognizing the κ light chain enhancer in B cell lymphocytes (Nishi *et al*., 2003). Active mammalian NF-κB consists of various hetero- or homodimer combinations of its five subunits. Two (p50, p52) of these subunits are responsible for DNA binding while three (p65, c-Rel, RelB) are involved with transactivation of the NF-κB molecule. The combination of subunits begets different binding affinities of a given activated NF-κB complex to a group of Rel homology domain (RHD) proteins with a related NF-κB DNA binding sites, all of which possess a common consensus sequence (Nishi *et al*., 2003).

In nonstimulated cells, NF-κB is complexed with the inhibitory protein IκBα. If bound to non-phosphorylated NF-κB, the NF-κB-IkBβ complex remains in the cytosol (Chen *et al*., 2003). However, if activated by phosphorylation of IkBβ, the NF-κB-IkBβ complex is translocated into the nucleus where it binds to the DNA of RHD proteins. In contrast, if bound to IκBα, the NF-κB-IκBα complex shuttles back and forth between the cytosol and nucleus but does not bind to RHD proteins.

With regard to the relative expression of NF-κB-controlled genes, it has been demonstrated (at least in testicular tissue) that sex-related Y-chromosome protein (SRY) can bind and activate transcription of IκBβ (Budde *et al*., 2002). An upregulation of IκBβ versus IκBα would result in a prolonged activation of NF-κB (Chen *et al*., 2003), and hence greater potential for expression of NF-κB-regulated genes. Recently, the promoter region of the SLC1A2 (GLT-1) gene has been cloned and was determined to contain several NF-κB binding domains (Su *et al*., 2003). Furthermore, promoter analysis using astroglial cells revealed that binding of activated NF-κB alone could transactivate SLC1A2, and resulted in elevation of GLT-1 mRNA, protein content, and of transport activity. Collectively, these observations suggest that processes that alter the expression of SRY likely will affect expression of SLC1A2, and hence GLT-1 expression levels.

The potential for a commonly shared component of the EGFR and NF-κB pathways in glial cells was suggested by the observation that simultaneous stimulation of both pathways yielded an additive response with regard to increased GLT-1 expression (Zelenaia *et al*., 2000). However, subsequent experimentation revealed that both EGFR- and cAMP-specific induction of GLT-1 expression was dependent on activation of phosphatidylinositol 3-kinase (PI3K), whereas stimulation of GLT-1 expression by NF-κB activation was not (Zelenaia *et al*., 2000). Because EGFR is a member of the RHD family, containing a promoter region with at least one NF-κB binding motif and several more in the regulatory region, the potential transactivation of EGFR gene transcription by activated NF-κB was investigated in an epithelial cell model (Nishi *et al*.,

2003). However, activated NF-κB (using several combinations of NF-κB subunits) failed to induce EGFR transcription.

The potential also appears to exist for regulation of GLT-1 expression by the relative content of GLT-1 in cell membranes. Specifically, a cytosolic protein (Ajuba) known to activate MAP kinase and to co-localize with GLT-1 in plasma membranes has been described (Marie *et al*., 2002). Ajuba does not appear to affect the binding affinity or transport capacity. Instead, it is thought that Ajuba may act as a GLT-1 transcriptional factor. That is, when the plasma membrane content of GLT-1 is too low to quantitatively bind Ajuba, Ajuba migrates to the nuclear membrane and initiates transcription of SLC1A2 (Marie *et al*., 2002).

Collectively, the above research suggests that EGFR and NF-κB activation pathways do not converge, at least not in glial-neuronal nor epithelial cell models. Determining whether these pathways are necessary and/or independently sufficient to control GLT-1 glutamate uptake is of great importance to biomedical researchers as decreased GLT-1 functional capacity in glial cell is integrally involved with several neurological diseases, including amyotrophic lateral sclerosis. Similarly, ruminant nutritionists need to have knowledge of those cellular signaling pathways that control EAAC1 and GLT-1 expression given the importance of glutamate uptake capacity to ruminant intermediary metabolism and the new knowledge that EAAC1 and GLT-1 expression by ruminant peripheral tissues is altered to support different commercially-important physiological states.

Coordinated expression and function relationships between GLT-1 and glutamine synthetase

Knowing whether GLT-1 expression is coordinated with that of glutamate-metabolizing enzymes is critical if we are to fully understand how the function of these high-affinity, high capacity transporters supports animal growth and physiology. GS catalyzes the ATP-dependent synthesis of glutamine from glutamate and ammonia. In at least rat skeletal muscle cells and/or tissue, GS transcription is stimulated by glutamine deprivation (Feng *et al*., 1990) or glucocorticoid challenge (Labow *et al*., 1999). Furthermore, it has recently been demonstrated that GS mRNA and activity can be upregulated by tumor necrosis factor-α (TNF-α) (Chakrabarti, 1998). Because GS mRNA levels were proportional to TNF-α challenge, and the rat GS gene contains a 5' TNF-α responsive element, it appears that expression of skeletal muscle GS by is at least partially regulated by TNF-α-initiated transcription.

In addition to transcriptional regulation, GS protein expression can also be regulated by mechanisms that alter GS protein stability. Specifically, if transcriptional-dependent increases in GS activity are not paralleled by an increased by removal of glutamine, then the elevated glutamine concentration will result in an accelerated rate of GS protein degradation, perhaps through the proteosome pathway (Labow *et al*., 2001).

Expression of GS by complex tissues (brain, kidney, liver, placenta) often is restricted to those cells responsible for mediating glutamine-glutamate cycles. For example, as discussed above, liver expression of GS protein (Cadoret *et al*., 2002) and activity (Gebhardt and Mecke, 1983)

is restricted to the small proportion of hepatocytes that surround the central vein (pericentral hepatocytes). This localization of GS matches that of ornithine aminotransferase (OAT) and system X^-_{AG} glutamate transport activities (Cadoret *et al*., 2002). Thus, GS activity is expressed in the cytosol of a population of hepatocytes that possesses both mitochondrial- and plasma-derived sources of glutamate substrate for amidation to glutamine.

β-catenin functions both as a structural component of adherens junctional complexes (intercellular adhesions) and as a nuclear transcription factor (Chesire and Isaacs, 2003). This latter role of β-catenin has been most well defined as its role as a mediator of membrane-to-nucleus signaling in the Wnt pathway (Micsenyi *et al*., 2004) and its interaction with the T-cell factor family, leading to stimulation of cell proliferation and/or inhibition of apoptosis (Calvisi *et al*., 2004). More specifically, it is now known that hepatocyte expression of GS, OAT, and GLT-1 is associated with the activation of Wnt/β-catenin signaling (Cadoret *et al*., 2002), and that GS is a specific target gene of β-catenin. Furthermore, unlike certain hepatocellular carcinomas, overexpression of GS and GLT-1 is specific to stimulation by β-catenin, not a non-specific response to heptocellular proliferation. In addition, at least in C6 glioma cells, GLT-1 expression can be stimulated by activation of the Wnt pathway (Palos *et al*., 1999). Thus, through its ostensible ability to coordinate OAT, GLT-1, and GS expression, β-catenin may be intimately involved in the regulation of hepatic ammonia scavenging, and therefore, whole-body N balance.

Posttranscriptional regulation of EAAC1 functional capacity

Although much progress has been made in establishing tissue-specific patterns of EAAC1 mRNA and protein expression, characterization of the promoter region for EAAC1 has not been reported. Accordingly, there is a relative paucity of knowledge about mechanisms responsible for initiating EAAC1 gene (SLC1A1) transcription, especially as compared to SLC1A2 (GLT-1). However, characterization of the posttranscriptional mechanisms that affect EAAC1 cellular content and functional capacity has revealed that posttranscriptional control of EAAC1 expression and function is complex. Figure 2 depicts the relationship of these mechanistic pathways.

From the literature, a prime candidate protein for controlling EAAC1-dependent glutamate uptake capacity by posttranscriptional mechanisms is protein kinase C (PKC), by altering the content of EAAC1 present in cell surface membranes. However, the data are mixed as to whether PKC stimulates or decreases EAAC1 activity. Specifically, activation of PKC stimulates trafficking of perinuclear vesicles of EAAC1 to the cell surface of C6 glioma cells (González *et al*., 2002), whereas activation of the PKC pathway in Xenopus oocytes overexpressing EAAC1 actually reduced EAAC1 activity and stimulated a shift in the distribution of EAAC1 from plasma to intracellular membranes vesicles (Trotti *et al*., 2001).

Although these data appear to be contradictory, they actually may be describing different cell models for PKC activation. That is, PKC "activity" is mediated by a family of PKC proteins consisting of three subgroups (Kazanietz *et al*., 2000). "Classic" PKC (cPKC; subtypes α, β, γ), is activated by diacylglycerols and phorbol esters, and requires Ca^{2+} as a cofactor. "Novel" PKC (nPKC, subtypes δ, ε, σ, η) also is activated by diacylglycerols and phorbol esters, but is Ca^{2+}-independent. The third PKC subgroup is "atypical" PKC (aPKC, subtypes ζ, λ) and is insensitive

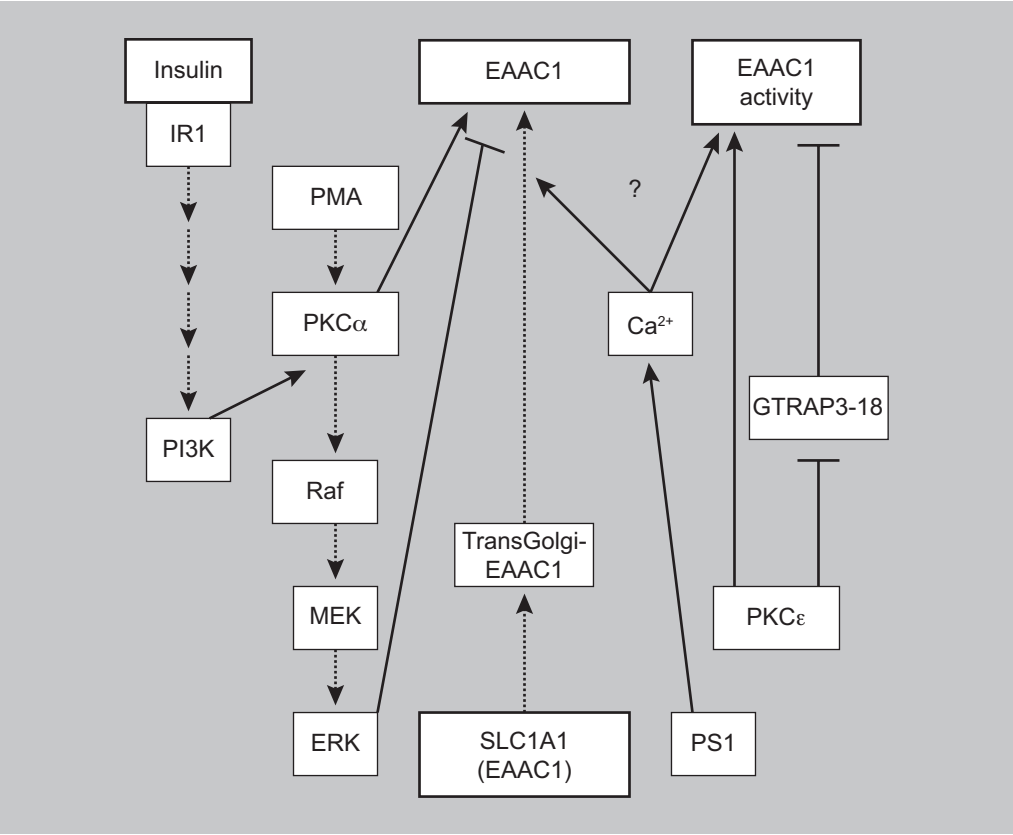

Figure 2. Working model for the posttranscriptional regulation of EAAC1 expression and activity. Pathway details and references are given in the text. Protein kinase C-α (PKCα) is expected to modulate EAAC1 translocation and presence in the plasma membrane, whereas PKCε-mediated events directly modulate EAAC1 activity. Presenilin 1 (PS1) maintains intracellular Ca^{++} levels, leading to the stimulation (solid arrows) of EAAC1 translocation and, perhaps, activity. In contrast, GTRAP3-18 binds plasma membrane EAAC1 and acts as an allosteric inhibitor (crossed solid lines).

to Ca^{2+} and to diacylglycerols and phorbol esters. Depending on the subgroup of PKC then, activation of PKC can either increase or decrease EAAC1-mediated glutamate uptake.

Common to all PKC-dependent alteration of EAAC1 membrane content is that inhibition of PI3K eliminates PKC-dependent increases in EAAC1 cell surface expression, but does not completely block glutamate uptake (González *et al*., 2002). This observation is consistent with the understanding that PI3K is an upstream activator of PKC. Because cPKC inhibition does not directly affect transporter function, PKCε, is the prime candidate for regulation of EAAC1 cell surface expression and activity.

Recently, a cytoplasmic glutamate transport associated protein (GTRAP3-18) has been shown to interact with the carboxy-terminal end of EAAC1 (Lin *et al*., 2001; Butchbach *et al*., 2002; Ikemoto *et al*., 2002). When associated with EAAC1, GTRAP3-18 functions as a negative allosteric modulator of glutamate uptake by EAAC1, thereby reducing the affinity of EAAC1 for its substrates (Lin *et al*., 2001). Transfection studies using human embryonic kidney cells revealed that EAAC1-mediated glutamate uptake was inversely proportional to GTRAP3-18 content (Butchbach *et al*., 2002), and that GTRAP3-18 has a high-affinity for EAAC1.

In terms of tissue expression patterns, GTRAP3-18 mRNA and protein have been found in the same tissues and regions of rat (Lin *et al*., 2001) and mouse (Butchbach *et al*., 2002; Ikemoto *et al*., 2002) tissues that express EAAC1, including the brain, heart, liver, kidney, and/or skeletal muscle. Although GTRAP3-18 expression and its potential effects on bovine or ovine EAAC1 has not been evaluated in ruminant models, at least one observation has been made that is consistent with the existence of a bovine GTRAP3-18 ortholog. Specifically, it is known that bovine EAAC1 mRNA content and functional activity both are upregulated in MDBK cells in response to amino acid deprivation (Nicholson and McGivan, 1996). Unexpectantly, a concomitant increase in EAAC1 protein content was not observed. However, if the amino acid deprivation treatment resulted in diminished GTRAP3-18 content, then the apparent discrepancy of unaltered EAAC1 protein content yet increased functional activity observed may have been explained.

Presenilin 1 (PS1) is an ubiquitously expressed polytopic transmembrane protein that is known for its roles of maintaining calcium homeostasis (Begley *et al*., 1999), protein trafficking (Naruse *et al*., 1998), and apoptosis (Mattson *et al*., 1998). Most recently, PS1 has been shown to be involved in the regulation of EAAC1 plasma membrane content, either through control of calcium or membrane trafficking (Yang *et al*., 2004). Specifically, the use of several PS1 neuronal depletion models revealed that loss of PS1-maintained intracellular Ca^{++} stores resulted in a decreased in EAAC1-mediated glutamate uptake activity and an acute reduction in plasma membrane EAAC1 content. However, it still remains to be determined whether PS1 regulates pre-formed EAAC1 expression through its effect on Ca^{++} stores-mediated events, and/or its interaction with EAAC1-specific trafficking proteins.

Modulation of chlortetracycline on cattle carcass quality and expression of system X^-_{AG} transporters and glutamine synthetase

Chlortetracycline has been used at subtherapeutic levels (typically, 350 mg/d) in diets of cattle for approximately 40 yr to promote "growth". Because the intestinal mucosa of CTC-fed cattle was "thinner" than non-treated cattle, the feeding of CTC was commonly thought to promote cattle growth through its antimicrobial effects on digestive tract microorganisms (Visek, 1978). Subsequently, it has been determined that CTC decreases intestinal epithelia mass, principally through a reduction in epithelial cell size, not number (Baldwin *et al*., 2000). Consequently, it is convenient to speculate that the lower energy (metabolic) costs of digesting and absorbing nutrients "spare" energy to support animal growth.

Early research indicated that CTC could also affect growth through the endocrine axis-controlled pathways (Landagora *et al*., 1957). This understanding led to the hypothesis that CTC produces

shifts in compositional gain through the alteration of growth factor profiles, including a reduction in thyroid releasing hormone and growth hormone by the pituitary (Rumsey *et al*., 1999, 2000) and decreased adiposity and lipogenic enzyme activity (McLeod *et al*., 2002). That CTC had effects on animal tissue (and not just gut flora) is consistent with tissue-level studies, which have revealed that CTC is absorbed by gut (Banerjee *et al*., 1976) and peripheral tissues (Kelly and Kanegis, 1967; Bodker *et al*., 1991; Gabler, 1991). Furthermore, that pituitary synthesis of thyroid stimulating hormone and growth hormone was reduced in CTC cattle is consistent with tissue-level research showing that single doses of super therapeutic levels (> 10 mg/kg BW) of CTC inhibit mammalian protein synthesis by intestinal (Greenberger, 1967; Niklov and Ilkov, 1961), and liver (Niklov and Ilkov, 1961). At the cellular level, early research suggested that the point of CTC action was prior to peptide bound formation (Clark and Chang, 1965).

Although the feeding of subtherapeutic levels of CTC to fattening cattle alters the amount and site of skeletal muscle and/or adipose tissue accretion, the mechanisms by which this nutrient repartitioning agent has on protein expression by these tissues and, more specifically, nutrient absorption capacity are unknown. Because we had previously demonstrated that the expression of GLT-1 and EAAC1 by tissues of fattening steers is sensitive to shifts in physiological load (Gissendanner *et al*., 2003b), and because CTC alters compositional gain (Baldwin *et al*., 2000), the potential alteration of GLT-1 or EAAC1 expression by CTC (350 mg/d) versus non-supplemented Angus-cross steers (BW = 350 ± 10 kg) fed a finishing diet (Kitts *et al*., 2004) for 112 d was evaluated (Sipe *et al*., 2004). CTC did not affect dry matter intake (DMI) nor gain:DMI, but did stimulate body weight gain/d and final body weight. In terms of carcass quality parameters, slaughter weight, carcass weight, longissimus dorsi area (longissimus area) area (cm^2), interstitial fat (marbling), and yield grade were all increased, whereas subcutaneous fat (longissimus fat cover), kidney/pelvic/heart fat % were decreased and bone maturity was not affected.

As noted above, intestinal epithelia, skeletal muscle, and adipose tissue are net users of blood glutamate. To evaluate the potential relationship between CTC-induced growth and carcass quality parameters and glutamate transport capacity, immunoblot analysis was used to determine the relative content of EAAC1 and GLT-1 in plasma membrane vesicles isolated from duodenal, jejunal, and ileal epithelia; liver; longissimus dorsi; subcutaneous, omental, and interstitial fat; as previously described by us (Howell *et al*., 2003). Because of the role of glutamate transport in support of GS by these tissues, the effect of CTC on GS content in liver, longissimus dorsi, and adipose tissue homogenates also was evaluated.

For the glutamate transporters, GLT-1 content of liver, longissimus dorsi, and subcutaneous fat was increased by 236%, 128%, and 524%, respectively, by CTC treatment, whereas EAAC1 was elevated (74%) only in omental fat tissue. In contrast to the stimulatory effect of CTC on glutamate transporter content, GS content was increased 220% in omental fat tissue but decreased greater than 15-fold in subcutaneous fat. When considered within a tissue, these results suggest that CTC-dependent upregulation of EAAC1 in omental fat supports glutamine synthetic capacity, and therefore N recycling. In contrast, the sole upregulation of GLT-1 by liver, longissimus dorsi, and subcutaneous fat tissues suggests that the effect of CTC in these tissues is to enhance carbon-metabolizing capacity.

The combination of these results, plus the understanding that binding of the GLT-1 gene (SCL1A2) by NF-κB is necessary and sufficient for expression of SCL1A2, strongly suggests that CTC mediates at least some of its effects by stimulating the binding of NFκB to its target genes. The identification of CTC-sensitive proteins in the NFκB activation pathway, and gene targets of NFκB, are current objectives of our research program.

Conclusions

High-affinity, highly concentrative, L-glutamate uptake is integral to support nitrogen and carbon homeostasis of many cell-types and tissue-level metabolic "cycles". In mammals, alteration of system X^-_{AG} transporter expression by non-CNS tissue is achieved through transcriptional and posttranscriptional mechanisms. Two system X^-_{AG} transport proteins, GLT-1 and EAAC1, are widely expressed by gastrointestinal tract and peripheral tissues of ruminants to support basal glutamate metabolism. Importantly, the transporter content is up-regulated by ileal epithelium and liver in response to dietary challenge, whereas transporter content of liver, white adipose, and longissimus dorsi tissue, but not intestinal epithelium, is altered as cattle develop from suckling through finishing physiological states. Furthermore, the content of GLT-1 of the liver, longissimus dorsi, and several adipose tissues was altered in response to daily feeding of subtherapeutic levels of chlortetracycline, whereas expression of GLT-1 by duodenal, jejunal, and ileal epithelia was not. Overall, these conclusions suggest that the demand for glutamate uptake capacity is less dynamic in intestinal epithelia than in liver, skeletal muscle, and fat tissues, tissues that use glutamate to regulate whole-body nitrogen and carbon metabolism in growing ruminants.

Acknowledgements

This research was supported by the University of Kentucky Exp. Res. Sta. and is a publication of the University of Kentucky Exp. Res. Station.

References

Arriza, J.L., S. Eliasof, M.P. Kavanaugh & S.C. Amara (1997). Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proceedings of the National Academy of Sciences of the U.S.A, 94, 4155-4160.

Baldwin, R.L., K.R. McLeod, T.H. Elasser, S. Kahl, T.S. Rumsey & M.N. Streeter (2000). Influence of chlortetracycline and dietary protein level on visceral organ mass of growing beef steers. Journal of Animal Science, 78, 3169-3176.

Ballatori, N., R.H. Moseley & J.L. Boyer (1986). Sodium gradient-dependent L-glutamate transport is localized to the canalicular domain of liver plasma membranes. Studies in rat liver sinusoidal and canalicular membrane vesicles. Journal of Biological Chemistry, 261, 6216-6221.

Banerjee, S. & K. Chakrabarti (1976). The transport of tetracyclines across the mouse ileum *in vitro*: the effects of cations and other agents. Journal of Pharmacy and Pharmacology, 28, 133-138.

Begley, J.G., W. Duan, S. Chan, K. Duff & M.P. Mattson (1999). Altered calcium homeostasis and mitochondrial dysfunction in cortical synaptic compartments of presenilin-1 mutant mice. Journal of Neurochemistry, 72, 1030-1039.

Begum, L., M.A. Jalil, K. Kobayashi, M. Iijima, M.X. Li, T. Yasuda, M. Horiuchi, A. del Arco, J. Satrustegui & T. Saheki (2002). Expression of three mitochondrial solute carriers, citrin, aralar1 and ornithine transporter, in relation to urea cycle in mice. Biochimica et Biophysica Acta, 1574, 283-292.

Biolo, G., X J. Zhang & R.R., Wolfe (1995). Role of membrane interorgan amino acid flow between muscle and small intestine. Metabolism, 44, 719-724.

Black, A.L., R.S. Anand, M.L. Bruss, C.A. Brown & J.A. Nakagiri (1990). Partitioning of amino acids in lactating cows: oxidation to carbon dioxide. Journal of Nutrition, 120, 700-710.

Bodker, A., T.B. Rasmussen & M.B. Christensen (1991). The absorption of chlortetracycline following transscrotal instillation for the treatment of primary hydrocele testis. Journal of Urology, 145, 792-794.

Bronson, J.T. (2000). Glutamate, at the interface between amino acid and carbohydrate metabolism. Journal of Nutrition, 130, 988S-990S.

Budde, L.M., C. Wu, C. Tilman, I. Douglas & S. Ghosh (2002). Regulation of IκBβ expression in testis. Molecular Biology of the Cell, 13, 4179-4194.

Burger, H.-J., R. Gebhardt, C. Mayer & D. Meckel (1989). Different capacities for amino acid transport in periportal and perivenous hepatocytes isolated by digitonin/collagenase perfusion. Hepatology, 9, 22-28.

Butchbach, M.E.R., L. Lai, G.C.-l. Lin (2002). Molecular cloning, gene structure, expression profile and functional characterization of the mouse glutamate transporter (EAAT3) interacting protein GTRAP3-18. Gene, 292, 81-90.

Cadoret, A., C. Overjero, B. Terris, E. Souil, L. Levy, W. H. Lamers, J. Kitajewski, A. Kahn & C. Perret (2002). New targets of β-catenin signaling in the liver are involved in the glutamine metabolism. Oncogene, 21, 8293-8301.

Calvisi, D.F., S. Ladu, V.M. Factor & S.S. Thorgeirsson (2004). Activation of β-catenin provides proliferative and invasive advantages in c-myc/TGF-α hepatocarcinogenesis promoted by phenobarbital. Carcinogenesis, 25, 901-908.

Cariappa, R. and M.S. Kilberg (1992). Plasma membrane domain localization, and transcytosis of the glucagon-induced hepatic system A carrier. American Journal of Physiology, 263, E1021-E1028.

Chakrabarti, R. (1998). Transcriptional regulation of the rat glutamine synthetase gene by tumor necrosis factor-α. European Journal of Biochemistry, 254, 70-74.

Chen, Y., J. Wu & G. Ghosh (2003). κB-Ras binds to the unique insert within the ankyrin repeat domain of IκBβ and regulates cytoplasmic retention of IκBβ•NFκB complexes. Journal of Biological Chemistry, 278, 23101-23106.

Chesire, D.R. & W.B. Isaacs (2003). β-catenin signaling in prostate cancer: an earlty perspective. Endocrine-Related Cancer, 10, 537-560.

Clark, J.M. and A.Y. Chang (1965). Inhibitors of the transfer amino acids from aminoacylsoluble ribonucleic acid to protein. Journal of Biological Chemistry, 240, 4734-4739.

Danbolt, N.C. (2001). Glutamate uptake. Progress in Neurobiology, 65, 1-105.

del Arco, A., J. Morcillo, J.R. Martinez-Morales, C. Galian, V. Martos, P. Bovolenta & J. Satrustegui (2002). Expression of the aspartate/glutamate mitochondrial carriers aralar1 and citrin during development and in adult rat tissues. European Journal of Biochemistry, 269, 3313-3320.

Duan, S., C.M. Anderson, B.A. Stein & R.A. Swanson (1999). Glutamate induces rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST. Journal of Neuroscience, 19, 10193-10200.

Fahey, G.C. & L.L. Berger (1988). In D.C. Church (ed.) The Ruminant Animal. Digestive Physiology and Nutrition. pp 269-297, Prentice-Hall, Inc., Englewood Cliffs, NJ.

Figiel, M., T. Maucher, J. Rozyczka, N. Bayatti & J. Engele (2003). Regulation of glial glutamate transporter expression by growth factors. Experimental Neurology, 183, 124-135.

Feng, B., S. K. Shiber & S. R. Max (1990). Glutamine regulates glutamine synthetase expression in skeletal muscle cells in culture. Journal of Cellular Physiology, 145, 376-380.

Frank, C., A.M. Giammarioli, L. Falzano, S. Rufini, S. Ceruti, A. Camurri, W. Malorni, M.P. Abbracchio & C. Fiorentini (2000). 2-Chloro-adenosine induces a glutamate-dependent calcium response in C2C12 myotubes. Biochemical and Biophysical Research Communications, 227, 546-551.

Frank, C., A.M. Giammarioli, L. Falzano, C. Fiorentini & S. Rufini (2002). Glutamate-induced calcium increase in myotubes depends on up-regulation of a sodium-dependent transporter. FEBS Letters, 527, 269-273.

Frayn, K.N., K. Khan, S.W. Coppack & M. Elia (1991). Amino acid metabolism in human subcutaneous adipose tissue in vivo. Clinical Science, 80, 471-474.

Gabler, W.L. (1991). Fluxes and accumulation of tetracyclines by human blood cells. Research Communications in Chemistry and Pathological Pharmacy, 72, 39-51.

Gaseously, G.C., V. Dall'Asta, O. Bussolati, M. Makowske & H.N. Christensen (1981). A stereoselective anomaly in dicarboxylic amino acid transport. Journal of Biological Chemistry, 256, 6054-6059.

Gebhardt, R. & D. Mecke (1983). Glutamate uptake by cultured rat hepatocytes is mediated by hormonally inducible, sodium-dependent transport systems. FEBS Letters, 161, 275-278.

Gegelashvili, G., M.B. Robinson, D. Trotti & T. Rauen (2001). Regulation of glutamate transporters in health and disease. Progress in Brain Research., 132, 267-286.

Gissendanner, S.J., N.M.P. Etienne & J.C. Matthews (2003a). Differential expression of EAAC1 and GLT-1 glutamate transporters by bovine epithelial tissues is not altered by physiological development. FASEB Journal, 17(5), A305.

Gissendanner, S.J., N.M.P. Etienne, K.R. McLeod & J.C. Matthews (2003b). The pattern of EAAC1 and GLT-1 glutamate transporter expression by skeletal muscle and adipose tissues of fattening cattle differs from that of glutamine synthetase. FASEB Journal, 17(5), A738.

Gonzalez, M.I., M.G. Kazanietz & M.B. Robinson (2002). Regulation of the neuronal glutamate transporter excitatory amino acid carrier-1 (EAAC1) by different protein kinase C subtypes. Molecular Pharmacology, 62, 901-910.

Greenberger, N.J. (1967). Inhibition of protein synthesis in rat intestinal slices by tetracycline. Nature, 214, 702-703.

Hack, V., O. Stutz, R. Kinscherf, M. Schykowski, M. Kellerer, E. Holm & W. Droge (1996). Elevated venous glutamate levels in (pre)catabolic conditions result at least partly from a decreased glutamate transport activity. Journal of Molecular Medicine, 74, 337-343.

Haussinger, D. and W. Gerok (1983). Hepatocyte heterogeneity in glutamate uptake by isolated perfused rat liver. European Journal of Biochemistry, 136, 421-425.

Haussinger, D., B. Stoll, T. Stehle & W. Gerok (1989). Hepatocyte heterogeneity in glutamate transport in perfused rat liver. European Journal of Biochemistry, 185, 189-195.

Hediger M.A. and T.C. Welbourne (1999). Introduction: glutamate transport, metabolism, and physiological responses. American Journal of Physiology, 277, F477-F480.

Heitmann, R.N., W.H. Hoover & C.J. Sniffen (1973). Gluconeogenesis from amino acids in mature wether sheep. Journal of Nutrition, 103, 1587-1593.

Heitmann, R.N. and E.N. Bergman (1981). Glutamate interconversions and glucogenicity in the sheep. American Journal of Physiology, 241, 465-472.

Holm, E., V. Hack, M, Tokus, R. Breitkreutz, A. Babylon, W. Droge (1997). Linkage between postabsorptive amino acid release and glutamate uptake in skeletal muscle tissue of healthy young subjects, cancer patients, and the elderly. Journal of Molecular Medicine, 75, 454-461.

Howell, J.A., A.D. Matthews, K.C. Swanson, D.L. Harmon & J.C. Matthews (2001). Molecular identification of high-affinity glutamate transporters in sheep and cattle forestomach, intestine, liver, kidney, and pancreas. Journal of Animal Science, 79, 1329-1336.

Howell, J.A., A.D. Matthews, K.C., Swanson, D.L. Harmon & J.C. Matthews (2003). Content of ileal EAAC1 and hepatic GLT-1 high-affinity glutamate transporters is increased in growing vs. nongrowing lambs, paralleling increased tissue D- and L-glutamate, plasma glutamine, and alanine concentrations. Journal of Animal Science, 81, 1030-1039.

Hundal, H.S., M.J. Rennie & P.W. Watt (1989). Characteristics of acidic, basic and neutral amino acid transport in the perfused rat hindlimb. Journal of Physiology (London), 408, 93-114.

Ikemoto, M.J., K. Inoue, S. Akiduki, T. Osugi, T. Imamura, N. Ishida & M. Ohtomi (2002). Identification of addicsin/GTRAP3-18 as a chronic morphine-augmented gene in amygdala. Molecular Neuroscience, 13, 2079-2084.

Kanai, Y. and M.A. Hediger (1992). Primary structure and functional characterization of a high-affinity glutamate transporter. Nature, 360, 467-471.

Kazanietz, M.G., M.J. Caloca, P. Eroles, T. Fujii, M.L. Garcia-Bermejo, M. Reilly & H. Wang (2000). Pharmacology of receptors for the phorbol ester tumor promoters: multiple receptors with different biochemical properties. Biochemistry and Pharmacology, 60, 1417-1424.

Kelly, R.G. & L.A. Kanegis (1967). Tissue distribution of tetracycline and chlortetracycline in the dog. Toxicology and Applied Pharmacology, 11, 114-120.

Kilberg, M.S., M.E. Handlogten & H.N. Christensen (1980) Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. Journal of Biological Chemistry, 255, 4011-4019.

Kilberg, M.S. and D. Haussinger (1992). Amino acid transport in liver. In: M.S. Kilberg and D. Haussinger (eds.) Mammalian Amino Acid Transport. Mechanisms and Control, Academic Press, San Diego, CA, pp. 133-148.

Kitts, S.E., J.C. Matthews, G.L. Sipe, T.S. Rumsey, T.H. Elasser, R.L. Baldwin & K.R. McLeod (2004). Effects of oral chlortetracycline and steroidal implant on growth performance and carcass merit of finishing beef steers. 2003 Kentucky Beef Cattle Report, pp. 5-7.

Kowalski, T.J. and M. Watford (1994). Production of glutamine and utilization of glutamate by rat subcutaneous adipose tissue in vivo. American Journal of Physiology, 266, E151-E154.

Kowalski , T.J., G. Wu & M. Watford (1997). Rat adipose tissue amino acid metabolism in vivo as assessed by microdialysis and arteriovenous techniques. American Journal of Physiology, 272, E613-E622.

Labow, B.I., W.W. Souba & S.F. Abcouwer (1999). Glutamine synthetase expression in muscle is regulated by transcriptional and posttranscriptional mechanisms. American Journal of Physiology 276 (Endocrinology and Metabolism 39), E1136-E1145.

Labow, B.I., W.W. Souba & S.F. Abcouwer (2001). Mechanisms governing the expression of the enzymes of glutamine metabolism-glutaminase and glutamine synthetase. Journal of Nutrition, 131, 2467S-2474S.

Landagora, F.T., L.L. Rusoff & B. Harris, Jr. (1957). Effect of chlortetracycline on carcass yields including physical and chemical composition of dairy calves. Journal of Animal Science, 16, 654-661.

Lange, K.H., J. Loresten, F. Isaksson, L. Simonsen, J. Bulow & M. Kaer (2002). Lipolysis in human adipose tissue during exercise: comparison of microdialysis and a-v measurements. Journal of Applied Physiology, 92, 1310-1316.

Levy, L.M., O. Warr & D. Attwell (1998). Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a chinese hamster ovary cell line selected for low endogenous Na^+-dependent glutamate uptake. Journal of Neuroscience, 18, 962-968.

Lin, C.I., I. Orlov, A.M. Ruggiero, M. Dykes-Hoberg, A. Lee, M. Jackson & J.D. Rothstein (2001). Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18. Nature, 410, 84-88.

Low, S.Y., P.M. Taylor, H.S. Hundal, C.. Pogson & M.J.Rennie (1992). Transport of L-glutamine and L-glutamate across sinusoidal membranes of rat liver. Effects of starvation, diabetes and corticosteroid treatment. The Biochemical Journal, 284, 333-340.

Low, S.Y., M.J. Rennie & P.M. Taylor (1994). Sodium-dependent glutamate transport in cultured rat myotubes increases after glutamine deprivation. FASEB Journal, 8, 127-131.

Marie, H., D. Billups, F.K. Bedford, A. Dumoulin, R.K. Goyal, G.D. Longmore, S.J. Moss & D. Attwell (2002). The amino terminus of the glial glutamate transporter GLT-1 interacts with the LIM protein Ajuba. Molecular and Cellular Neurosciences, 19, 152-164.

Matsuo, H., Y. Kanai, J.Y. Kim, A. Chairoungdua, D.K. Kim, J. Inatomi, Y. Shigeta, H. Ishimine, S. Chaekuntode, K. Tachampa, H.W. Choi, E. Babu, J. Fukuda & H. Endou, (2002). Identification of a novel Na^+-independent acidic amino acid transporter with structural similarity to the member of a heterodimeric amino acid transporter family associated with unknown heavy chains. Journal of Biological Chemistry, 277, 21017-21026.

Matthews, J.C., M.J. Beveridge, M.S. Malandro, J.D. Rothstein, M. Campbell-Thompson, J.W. Verlander, D.A. Novak & M.S. Kilberg (1998). Activity and protein localization of multiple glutamate transporters in gestation day 14 vs. day 20 rat placenta. American Journal of Physiology, 274, C603-C614.

Matthews, J.C., M.J. Beveridge, E. Dialynas, A. Bartke, M.S.Kilberg & D.A. Novak (1999). Placental anionic and cationic amino acid transporter expression in growth hormone overexpressing and null IGF-II or null IGF-I receptor mice. Placenta, 20, 639-650.

Matthews, J.C. (2000). Amino Acid and Peptide Transport Systems. In: J.P.F. D'Mello (ed.) Farm Animal Metabolism and Nutrition. pp 3-23, CABI Publishing, New York.

Mattson, M.P., Q. Guo, K. Furukawa & W.A. Pedersen (1998). Presenilins, the endoplasmic reticulum, and neural apopotosis in Alzheimer's disease. Journal of Neurochemistry, 70, 1-14.

McLeod, K.R., R.L. Baldwin, M.B. Solomon, A.V. Capuco & D.L. Harmon (2002). Influence of ruminal and postruminal carbohydrate infusion on visceral organ mass and adipose tissue accretion in growing beef steers. Journal of Animal Science, 80 (suppl. 1), 952.

Meijer, A.J., W.H. Lamers & R.A. Chamuleau (1990). Nitrogen metabolism and ornithine cycle function. Physiology Reviews, 70, 701-748.

Micsenyi, A., X. Tan, T. Sneddon, J.H. Luo, G.K. Michalopoulos & S.P. Monga (2004). Beta-catenin is temporally regulated during normal liver development. Gastroenterology, 126, 1134-1146

Moores, R.R., P.R. Vaughn, F.C. Battaglia, P.V. Fennessey, R.B. Wilkening & G. Meschia (1994). Glutamate metabolism in fetus and placenta of late-gestation sheep. American Journal of Physiology, 267, R89-R96.

Moorman, A.F., J.L. Vermeulen, R. Charles & W.H. Lamers (1989). Localization of ammonia-metabolizing enzymes in human liver: ontogenesis of heterogeneity. Hepatology, 9, 367-372.

Moorman, A.F., P.A. de Boer, A.T. Das, W.T. Labruyere, R. Charles & W.H. Lamers (1990). Expression patterns of mRNAs for ammonia-metabolizing enzymes in the developing rat: the ontogenesis of hepatocyte heterogeneity. The Histochemial Journal, 22, 457-468.

Munir, M., D.M. Correale & M.B. Robinson (2000). Substrate-induced up-regulation of Na^+-dependent glutamate transport activity. Neurochemistry International, 37, 147-162.

Naruse, S., G. Thinakaran, J.J. Luo, J.W. Kusiak, T. Tomita, T. Iwatsubo, X. Qian, D.D. Ginty, D.L. Price, D.R. Borchelt, P.C. Wong, S.S. Sisodia (1998). Effects of PS1 deficiency on membrane protein trafficking in neurons. Neuron, 21, 1213-1221.

Newsholme, E.A. & M. Parry-Billings (1990). Properties of glutamate release from muscle and its importance for the immune system. Journal of Parental and Enteral Nutrition, 14, 63S-67S.

Nicholson, B. & J.D. McGivan (1996). Induction of high affinity glutamate transport activity by amino acid deprivation in renal epithelial cells does not involve an increase in the amount of transporter protein. Journal of Biological Chemistry, 271, 12159-12164.

Niklov, T.K. and A.T. Ilkov (1961). Effect of chlortetracycline on methionine-^{35}S incorporation into macroorganism proteins. International Congress of Biochemistry, 2 (Abstr.), 102.

Nishi, H., G. Neta, K.H. Nishi, L.M. Akers, T. Rikiyama, K.N. Proctor, B.A. Murphy & A.C. Johnson (2003). Analysis of the epidermal growth factor receptor promoter: the effect of nuclear factor-κB. International Journal of Molecular Medicine, 11, 49-55.

Nissim, I. (1999). Newer aspects of glutamine/glutamate metabolism: the role of acute pH changes. American Journal of Physiology, 277, F493-F497.

Novak, D., F. Quiggle, C. Artime & M. Beveridge (2001). Regulation of glutamate transport and transport proteins in a placental cell line. American Journal of Physiology Cell Physiology, 281, C1014-C1022.

Palos, T.P., S. Zheng & B.D. Howard (1999). Wnt signaling induces GLT-1 expression in rat C6 glioma cells. Journal of Neurochemistry, 73, 1012-1023.

Patterson, B.W., J.F. Horowitz, G. Wu, M. Watford, S.W. Coppack & S. Klein (2002). Regional muscle and adipose tissue amino acid metabolism in lean and obese women. American Journal of Endocrinology Metabolism, 282, E931-E936.

Peghini, P., J. Janzen & W. Stoffel (1997). Glutamate transporter EAAC-1-deficient mice develop dicarboxylic aminoaciduria and behavioral abnormalities but no neurodegeneration. EMBO Journal, 16, 3822-3832.

Plakidou-Dymock, S. and J.D. McGivan (1993). Regulation of the glutamate transporter by amino acid deprivation and associated effects on the level of EAAC1 mRNA in the renal epithelial cell line NBL-I. The Biochemical Journal, 295, 749-755.

Reeds, P.J., D.G. Burrin, F. Jahoor, L. Wykes, J. Henry & E.M Frazer (1996). Enteral glutamate is almost completely metabolized in first pass by the gastrointestinal tract of infant pigs. American Journal of Physiology, 270, E413-E418.

Rennie, M.J., J.L. Bowtell, M. Bruce & S.E.O. Khogali (2001). Interaction between glutamate availability and metabolism of glycogen, tricarboxylic acid cycle intermediates and glutathione. Journal of Nutrition, 131, 2488S-2490S.

Revest, P.A. and P.F. Baker (1988). Glutamate transport in large muscle fibres of Balanus nubilus. Journal of Neurochemistry, 50, 94-102.

Ritchie, J.W., F.E. Baird, G.R. Christie, A. Stewart, S.Y. Low, H.S. Hundal & P.M. Taylor (2001). Mechanisms of glutamine transport in rat adipocytes and acute regulation by cell swelling. Cell Physiology and Biochemistry, 11, 259-270.

Robinson, M.B. (1999). The family of sodium-dependent glutamate transporters: a focus on the GLT-1/EAAT2 subtype. Neurochemistry International, 33, 479-491.

Rolinski, B., F.A. Baumeister & A.A. Roscheer (2001). Determination of amino acid tissue concentrations by microdialysis: method evaluation and relation to plasma values. Amino Acids, 21, 129-138.

Rumsey, T.S., A.C. Hammond, T.H. Elasser (1999). Effects of oral chlortetracycline and dietary protein level on plasma concentrations of growth hormone and thyroid hormone in beef steers before and after challenge with a combination of thyrotropin-releasing hormone and growth-hormone releasing hormone. Journal of Animal Science, 77, 2079-2087.

Rumsey, T.S., K. McLeod, T.H. Elasser, S. Kahl & R.L. Baldwin (2000). Responses to estrogenic growth promoter in beef steers fed varying nutritional regimens. Journal of Animal Science, 78, 2765-2770.

Schlag, B.D., J.R. Vondrasek, M. Munir, A. Kalandadze, O.A. Zelenaia, J.D. Rothstein & M.B. Robinson (1998). Regulation of the glial Na^+-dependent glutamate transporters by cyclic AMP analogs and neurons. Molecular Pharmacology, 53, 355-369.

Schluter, K.M. Figiel, J. Rozcyzka & J. Engele (2002). CNS region-specific regulation of glial glutamate transporter expression. European Journal of Neuroscience, 16, 836-842.

Sims, K. D., D. J. Straff & M. B. Robinson (2000), Platelet-derived growth factor rapidly increases activity and cell surface expression of the EAAC1 subtype of glutamate transporter through activation of phosphatidylinositol 3-kinase. Journal of Biological Chemistry, 274, 5228-5237.

Sipe, G.L., S.E. Kitts, K.R. McLeod & J.C. Matthews (2004). Chlortetracycline, but not synovex-S, differentially affects tissue expression of high-affinity glutamate transporters in fattening steers. Journal of Animal Science, 82 (suppl. 1), 42.

Stoll, B., S. McNelly, H.P. Butcher & D. Haussinger (1991). Functional hepatocyte heterogeneity in glutamate, aspartate and α-ketoglutarate uptake: a histoautoradiographical study. Hepatology, 13, 247-253.

Su, Z.Z., M. Leszczyniecka, D.C. Kang, D. Sarkar, W. Chao, D.J. Volsky & F.B. Fisher (2003). Insights into glutamate transport regulation in human astrocytes: cloning of the promoter for excitatory amino acid transporter 2 (EAAT2). Proceedings of the National Academy of Science of the USA, 100, 1955-60.

Summers, L.K., P. Arner, V. Ilic, M.L. Clark, S.M. Humphreys & K.N. Frayn (1998). Adipose tissue metabolism in the postprandial period: microdialysis and arteriovenous techniques compared. American Journal of Physiology, 274, E651-655.

Tagari, H. and E.N. Bergman (1978). Intestinal disappearance and portal blood appearance of amino acids in sheep. Journal of Nutrition, 108, 790-803.

Tanaka, K. and T. Welbourne (2001). Enhanced ammonium excretion in mice lacking the glutamate transporter GLT-1. Journal of Articles in Support of the Null Hypothesis, 108, A0287.

Taylor, P.M. and M.J. Rennie (1987). Perivenous localisation of Na-dependent glutamate transport in perfused rat liver. FEBS Letters, 221, 370-374.

Trotti, D., J.B. Peng, J. Dunlop & M.A. Hediger (2001). Inhibition of the glutamate transporter EAAC1 expressed in Xenopus oocytes by phorbol esters. Brain Research, 914, 196-203.

Ushmorov, A., V. Hack & W. Druge (1999). Differential reconstitution of mitochondrial respiratory chain activity and plasma redox state by cysteine and ornithine in a model of cancer cachexia. Cancer Research, 59, 3527-3534.

van Milgen, J. (2002). Modeling biochemical aspects of energy metabolism in mammals. Journal of Nutrition, 132, 3195-2002.

Vaughn, P.R., C. Lobo, F.C. Battaglia, P.V. Fennessey, R.B. Wilkening & G. Meschia (1995). Glutamine-glutamate exchange between placenta and fetal liver. American Journal of Physiology, 268, E705-E711.

Vesli, R.F., M. Klaude, O.E. Rooyackers, I. Tjader, H. Barle & J. Wernerman (2002). Longitudinal pattern of glutamine/glutamate balance across the leg in long-stay intensive care patients. Clinical Nutrition, 21, 505-514.

Visek, W.J. (1978). The mode of growth promotion by antibiotics. Journal of Animal Science, 46, 1447-1469.

Wagenaar, G.T., W.J. Geerts, R.A. Chamuleau, N.E. Deutz & W.H. Lamars (1994). Lobular patterns of expression and enzyme activities of glutamine synthetase, carbamoylphosphate synthase and glutamate dehydrogenase during postnatal development of the porcine liver. Biochimica et Biophysica Acta, 1200, 265-270.

Watford, M. (2000). Glutamine and glutamate metabolism across the liver sinusoid. Journal of Nutrition, 130, 983S-987S.

Welbourne, T.C. and J.C. Matthews (1999). Glutamate transport and renal function. American Journal of Physiology, 277, F501-F505.

Welbourne, T. and I. Nissim (2001). Regulation of mitochondrial glutamine/glutamate metabolism by glutamate transport: studies with ^{15}N. American Journal of Physiology Cell Physiology, 280, C1151-C1159.

Welbourne, T., R. Routh, M. Yudkoff & I. Nissim (2001). The glutamine/glutamate couplet and cellular function. News in Physiological Sciences, 16, 157-160.

Wu, G. (1998). Intestinal mucosal amino acid catabolism. Journal of Nutrition, 128, 1249-1252.

Yang, Y., G.A. Kinney, W.J. Spain, J.C.S. Breitner & D.G. Cook (2004). Presenilin-1 and intracellular calcium stores regulate neuronal glutamate uptake. Journal of Neurochemistry, 88, 1361-1372.

Zerangue, N. and M.P. Kavanaugh (1996). Flux coupling in a neuronal transporter. Nature, 383, 634-637.

Zelenaia, O., B.D. Schlag, G.E. Gochenauer, R. Ganel, W. Song, J.S. Beesley, J.B. Grinspan, J.D. Rothstein & M.B. Robinson (2000). Epidermal growth factor receptor agaonists increase expression of glutamate transporter GLT-1 in astrocytes through pathways dependent on phosphatidylinositol 3-kinase and transcription factor NF-κB. Molecular Pharmacology, 57, 667-678.

Characterisation and nutritional regulation of the main lipogenic genes in the ruminant lactating mammary gland

L. Bernard, C. Leroux and Y. Chilliard
Adipose Tissue and Milk Lipid Laboratory, Herbivore Research Unit, INRA-Theix, 63 122 St-Genès-Champanelle, France

Abstract

The effect of nutrition on milk fat yield and composition has been widely investigated in cows and goats, with similar responses for fatty acid (FA) composition and marked species-differences in milk fat yield response. Nevertheless, a few *in vivo* studies focused on the effect of nutrition on mammary lipogenic gene expression, in cows (mainly fed milk fat depressing diets) and goats (fed lipid supplemented diets), thanks to the recent characterisation of these genes in ruminant species. These studies showed some similarities in the regulation of gene expression between the 2 species, although these responses were not always in accordance with milk FA secretion responses. A central role for *trans*-FA as regulators of milk fat synthesis has been proposed, with *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA as putative candidates for a milk fat depressing effect. The milk *trans*-10 C18:1 percentage was negatively correlated with milk fat yield response in goat and cow, despite the fact that in goat the fat yield response was always positive and related in part to stearic acid concentration. Milk *trans*-10, *cis*-12 CLA is sometimes correlated with milk fat depression in cows and, when post-ruminally infused, involved in a coordinate reduction in mRNA abundance of key lipogenic enzymes. The molecular mechanisms involved in the nutritional regulation of gene expression are not yet well identified but a putative central role for SREBP-1 has been outlined as mediator of FA effects, whereas the roles of PPARs and Stat5 need to be unravelled. It is expected that more complete identification of the inhibitors and activators of fat synthesis, and better knowledge of cellular mechanisms involved, thanks to the development of *in vitro* functional systems for lipid synthesis and secretion, will allow future progress in understanding differences between ruminant species, and a better control of milk fat yield and FA composition.

Keywords: nutrition, gene expression, lipogenesis, mammary gland, lactating ruminant

Introduction

The major constituents of milk (lipids, proteins, carbohydrates and salts), and their concentrations can be affected by physiological, genetic, nutritional and environmental factors. In ruminants, two major factors among these determine milk composition: the genetic factor through the animal species, and nutritional factors. Nutrition has a considerable effect on the composition of the lipids (Jensen, 2002), whereas the composition of the protein fraction is generally little affected by this factor (Coulon *et al*., 2001). Moreover, milk fat is an important component of the nutritional quality of dairy products, since saturated fatty acids (FA) (mainly C12, C14 and C16) are often considered to exert a negative effect on human health if consumed in excess (Williams, 2000), while other FA could exert positive effects such as, in the case of oleic and

linolenic acids, by a direct vascular antiatherogenic action (Massaro *et al*., 1999). Furthermore, conjugated linoleic acid (CLA), another FA found in ruminant products including milk, was shown recently, in rodents, to have a variety of beneficial effects, such as anticarcinogenic action, immune system enhancement, etc…(Pariza *et al*., 2001). Additionally, milk fat content and FA composition contribute significantly to technological and sensorial qualities of dairy products. So modification of milk fat content and FA composition by dietary manipulation has been investigated comprehensively in cows and goats. Particularly the effect of fat supplementation of the diet has been investigated because of its efficiency in modifying milk FA composition.

The consequences of lipid supplementation on the milk yield and fat and protein contents are well known, in particular in bovines: an increase in milk production (with most lipid supplements), and a slight but systematic reduction in the protein and casein contents. In dairy cows the supplements given have not all the same efficiency with respect to fat content modulation, due to important interactions between dietary forages and concentrates, and their components (fibres, starch, lipids, etc.). Thus, decreases in fat content are recorded with concentrate-rich diets, concentrate-rich diets supplemented with vegetable oils, and diets supplemented with fish oil, while a large increase is noted with encapsulated lipids (Chilliard and Ferlay, 2004; Palmquist *et al*., 1993). In contrast, in goats, almost all types of lipid supplements induce a marked increase in the milk fat content without systematic modification of milk production or protein content (Chilliard *et al*., 2003a). These modifications in the quantity of fat secreted are generally accompanied by an important modification of the milk FA composition which is well documented both in cows (Chilliard *et al*., 2000, 2001; Palmquist *et al*., 1993) and goats (Chilliard *et al*., 2003a). For example, supplementation with linseed oil significantly increases the CLA, *trans*-FA and C18:3 (n-3) percentages and markedly reduces that of C10 to C16 saturated FA in goat's (Rouel *et al*., 2004) and cow's (Loor *et al*., 2005a) milk while the short-chain FA remain unchanged (C6 and C8) or tend to increase (C4).

The mechanisms underlying these intra- and inter-species responses are not yet well understood. However, over the past five years, thanks to the characterisation of the lipogenic genes involved in milk synthesis and secretion associated with the development of molecular biology tools, studies have been undertaken at molecular level in order to relate the effects of diet on the milk FA profile to changes in mammary gland lipid metabolism. These studies focused on candidate genes involved in lipogenesis (mainly genes for the enzymes involved in uptake, *de novo* synthesis, desaturation and esterification of FA) in order to evaluate the effects of the diet on the abundance of their transcripts and/or enzymatic activities. The aim of this paper is to review (i) the recent characterisation of the main lipogenic genes (ii) the known effects of nutritional factors, and particularly those of fat supplementation, on ruminant mammary lipogenic gene expression, in relation with milk fat content and FA composition and (iii) the putative molecular mechanisms underlying these regulations.

Origin of milk fatty acids

Milk fat comprises *ca.* 98% of triglycerides whose FA represent *ca.* 95%, less than 1% of phospholipids, and small amounts of cholesterol, 1,2 diacylglycerol, monoacylglycerol and free FA. FA's have two main origins. Short and medium chain FA (C4:0 to C16:0) represent 40-50%

of the FA secreted in milk, and are almost (except *ca.* one half of the C16:0) exclusively the result of *de novo* synthesis in the mammary gland (Figure 1) from acetate and 3-hydroxybutyrate, produced by ruminal fermentation of carbohydrates and by rumen epithelium from absorbed butyrate, respectively. Long-chain FA (≥ C18) are imported from the plasma, where they are either released by the enzyme lipoprotein lipase (LPL) (Barber *et al*., 1997) from triglycerides circulating in chylomicra or very low density lipoprotein (VLDL), or derived from the plasma non-esterified fatty acids (NEFA) that circulate bound to albumin. These FA originate mainly from the absorption of dietary lipids from the digestive tract (with the dietary FA undergoing total or partial hydrogenation in the rumen) and from the mobilisation of body reserves (lipomobilisation, especially at the beginning of lactation) . Typically, mobilisation of body fat accounts for less than 10% of milk FA but this proportion increases in ruminants in negative energy balance in direct proportion to the extent of the energy deficit (Bauman and Griinari, 2001; Chilliard *et al*., 2003a). In addition, FA may be desaturated but not elongated in the secretory mammary epithelial cells (MEC) (Chilliard *et al*., 2000).

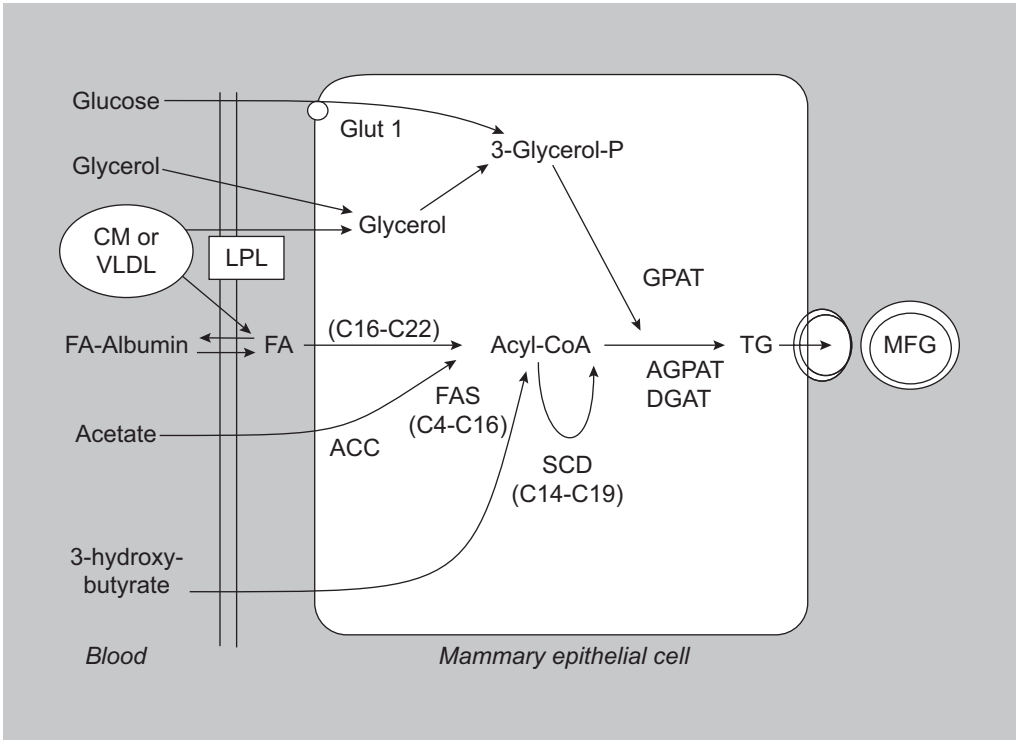

Figure 1. Milk fat synthesis in the ruminant mammary epithelial cell (Source: Chilliard et al.*, 2000).*
Abbreviations used: ACC=acetyl-CoA carboxylase, AGPAT=acyl glycerol phosphate acyl transferase, CM=chylomicron, DGAT=diacyl glycerol acyl transferase, FA=fatty acid, FAS=fatty acid synthase, Glut 1=glucose transporter 1, GPAT=glycerol-3 phosphate acyl transferase, LPL=lipoprotein lipase, MFG=milk fat globule, SCD=stearoyl-CoA desaturase, TG=triglyceride, VLDL=very low density-lipoprotein.

Characterisation of the main lipogenic genes and tools for studying gene expression and regulation

Within the mammary tissue, the metabolic pathway for *de novo* FA synthesis involves two key enzymes: acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), while LPL is involved in the uptake of plasma FA. These FA could then be desaturated by the SCD resulting in synthesis of *cis*-9 unsaturated FA. In a final step, the FA are esterified to glycerol sequentially *via* glycerol-3 phosphate acyl transferase (GPAT), the acyl glycerol phosphate acyl transferase (AGPAT) and diacyl glycerol acyl transferase (DGAT), and the triglycerides are secreted as milk fat globules (Figure 1). These enzymes involved in the key processes of lipogenesis within the mammary gland, are considered as the major lipogenic enzymes, and the genes specifying them are candidate genes whose regulation has been studied first.

In ruminants, the recent knowledge of the cDNA sequences of several lipogenic genes, LPL (Bonnet *et al*., 2000a; Senda *et al*., 1987), ACCα (Barber and Travers, 1998; Mao *et al*., 2001), FAS (Leroux *et al*., submitted), SCD (Bernard *et al*., 2001; Ward *et al*., 1998), DGAT (Winter *et al*., 2002), AGPAT (Mistry and Medrano, 2002) has allowed the development of molecular tools for studying their expression, through the quantification of their mRNA, by Northern Blot or real-time RT-PCR, and/or through the determination of the activity of the corresponding enzymes.

Uptake of fatty acids

LPL catalyses the hydrolysis of lipoprotein triacylglycerol, selectively releasing FA esterified at the *sn*-1 (-3) position. The bovine mammary tissue expresses three LPL transcripts of 1.7, 3.4 and 3.6 kb in size (Kirchgessner *et al*., 1987; Senda *et al*., 1987) by the alternative use of polyadenylation site with a predominance of the 3.4 kb transcript in the mammary gland (Senda *et al*., 1987) as in adipose tissue and of the 3.6 kb transcript in muscles (Bonnet *et al*., 2000b). The total cDNA sequence in ovine species has been reported by Bonnet *et al.* (2000a). LPL is subjected to complex regulation by dietary and hormonal factors, which modulate LPL activity *via* transcriptional, post-transcriptional, and post-translational mechanisms. Mammary LPL activity increases markedly immediately prior to parturition and remains high throughout lactation, whereas it is simultaneously down-regulated in adipose tissue in cows (Shirley *et al*., 1973) and goats (Chilliard *et al*., 2003a).

A number of immunochemical and biochemical studies have indicated that LPL is located, both on (or near) the surface and within the cell of the major cell-type of the different tissues as well as on the luminal surface of vascular endothelial cells. Mammary tissue contains various cell types in addition to parenchymal secretory MEC. These include adipocytes, in varying proportions to MEC depending on the developmental and physiological status of the mammary gland. From histological studies made on rodent mammary gland, discrepancies with respect to LPL localisation were reported. In rodents, some studies (Camps *et al*., 1990) concluded that the origin of mammary LPL is the secretory MEC while others (Jensen *et al*., 1991; Neville and Picciano, 1997) concluded that mammary LPL could originate at least in part from mammary adipocytes and is subsequently secreted and transported by cellular uptake and transcytosis, both to its final site of action on the capillary endothelial cell and through the secretory MEC

into milk. Indeed, Jensen *et al.* (1991) located LPL protein and mRNA in mammary interstitial cells which are depleted adipocytes or adipocyte precursors. Conversely, Camps *et al.* (1990) demonstrated by *in situ* hybridisation and immunofluorescence the presence in MEC of LPL mRNA and protein, respectively, with a loss of the observed signals after no suckling for 12 h, suggesting a regulation of mammary LPL at the gene expression level.

Protein LPL release into milk and its putative physiological function remains, however, unresolved questions. In lactating ruminants, milk LPL activity is lower during early and late lactation than during mid lactation, is decreased during fasting and is positively correlated to visceral adipose tissue LPL activity (review by Chilliard *et al.*, 2003a). Thus, part of the milk enzyme could originate from adipose tissue. However, milk LPL activity is decreased by reducing milking frequency, which suggests that increased intra-mammary pressure could slow down mammary LPL secretion. The complexity of mammary/milk LPL regulation is also illustrated by the facts (review by Chilliard *et al.*, 2003a) that milk LPL activity is much lower and less bound to caseins micelles in goat than in cow, and that milk LPL activity is higher in goats presenting αs1-casein variants which are linked to lower milk casein and fat contents. Furthermore, post-milking lipolysis is better correlated to LPL activity in goat than in cow milk, and this activity plays an important role in the development of the typical flavour of goats' dairy products.

Studies in lactating goats with measurements of arteriovenous differences have shown that the utilisation of substrates (triglycerides, NEFA) for milk lipid synthesis is related to their plasma concentrations (Annison *et al.*, 1968). Other experiments demonstrated that the availability of the substrate determines its utilisation by the mammary gland with either no net utilisation of NEFA at plasma concentration below 0.2 mM (Nielsen and Jakobsen, 1994), or a large NEFA utilisation when plasma triglycerides are low and NEFA are high, as in fasting animals (West *et al.*, 1972). Conversely, triglyceride utilisation increases when its plasma concentration increases, for example after duodenal lipid infusion (Gagliostro *et al.*, 1991), which results in mammary net production of NEFA, due to their release in the vascular bed during LPL action (Figure 1).

The mechanism by which FA cross the capillary endothelium and interstitial space to reach the MEC has not been identified. Once at the MEC, FA could cross the plasma membrane by diffusion or via a saturable transport system. In mammals, an important role in regulating transport and concentration of FA in the cytosol has been reported for the acyl-CoA binding proteins (ACBP) (Knudsen *et al.*, 2000), that bind long-chain acyl-CoA. However, in ruminants, concentrations of ACBP were found to be lower in the mammary gland and muscles compared to those found in liver cytosol (Mikkelsen and Knudsen, 1987). Elsewhere, it has been suggested in rodents and ruminants that another FA binding protein expressed in the lactating MEC is CD36 (Cluster of Differentiation 36), a protein of the milk fat globule membrane also found in heart, platelets and adipocytes, which may function as a transporter of long-chain FA (Abumrad *et al.*, 1993; Mather, 2000). The detection of CD36 in mammary tissue could be linked to the presence of adipocytes. However, the presence of CD36 mRNA in rodent MEC lines has been shown, as well as a slight enhancement of its gene expression by treatment with lactogenic hormones (Aoki *et al.*, 1997).

Moreover, fatty acid binding proteins (FABP), a family of intracellular lipid binding proteins found across numerous species including mammals, are involved in the uptake and intracellular

trafficking of FA in many tissues (Lehner and Kuksis, 1996; Makowski and Hotamisligil, 2004). In the bovine mammary gland, co-expression of FABP and CD36 has been shown; this reaches its maximum during lactation and declines during involution (Spitsberg *et al.*, 1995) demonstrating that their expression is related with physiological state and the level of lipid transport and metabolism within the cell. Barber *et al.*, (1997) proposed a role for CD36 in the transport of FA across the secretory MEC membrane, working in conjunction with intracellular FABP. Moreover, the coexistence of two forms of FABP has been demonstrated (Specht *et al.*, 1996) in the bovine lactating mammary gland, identified as A-FABP and H-FABP, thus named according to the tissue of their first isolation, adipose tissue and heart, respectively. Looking at the proteins' cellular location, Specht *et al.* (1996) showed that A-FABP was present in myoepithelial cells, whereas H-FABP was detected in MEC. Expression of FABP types is generally interpreted in terms of specialised functions in FA metabolism. H-FABP is predominantly found in cells where FA are used as energy source and is probably involved in their β-oxidation. The significance of its abundance in the mammary gland, which has active triglyceride synthesis and low FA oxidation during lactation, remains to be understood.

De novo fatty acid synthesis

Acetyl-CoA carboxylase (ACC)

Two principal isoenzymes of ACC have been described, which are transcribed from separate genes termed ACCα and ACCβ. The ACCα gene encodes a 265 kDa enzyme which is ubiquitously expressed but found at its highest levels in lipogenic tissues (Lopez-Casillas *et al.*, 1991). The ACCβ gene encodes a 280 kDa enzyme which is the main isoenzyme expressed in heart and skeletal muscles (Abu-Elheiga *et al.*, 1997). The tissue distribution of these isoenzymes, together with the demonstration that the α-isoform was found in the cytoplasm and the β-isoform associated to the outer membrane of mitochondria (Abu-Elheiga *et al.*, 2000), led to the suggestion that they have different functions: ACCα would provide cytoplasmic malonyl-CoA for the synthesis of FA, while malonyl-CoA generated by ACCβ would take part in the regulation of carnitine palmitoyl-CoA transferase I activity, and therefore the rate of FA β-oxidation. The regulation of both ACC isoenzymes is extremely complex and occurs at the protein catalytic efficiency level through allosteric modification by cellular metabolites possessing a positive (citrate) or negative effect (malonyl-CoA and long-chain acyl-CoA), as well as by covalent reversible modification *via* phosphorylation/dephosphorylation under hormonal control.

In ruminants, the ACCα cDNA sequence was first reported by Barber and Travers (1995) and corresponds to the synthesis of a protein with 2346 amino acids. More recently, ACCα gene was characterised in sheep (Barber and Travers, 1998) and cattle (Mao *et al.*, 2001). The existence of three promoters, PI, PII and PIII, has been demonstrated. The promoters generate multiple transcripts differing in the sequence of their 5'UTR. This mRNA multiplicity is due to both the use of the three promoter systems, and alternative splicing of the primary transcripts from promoter I and II, thus resulting in 5 classes of transcripts. The three promoters are used in tissue-differential fashion. PIII is limited to lung, liver, kidney, brain and predominantly the mammary gland during lactation in bovine (Mao *et al.*, 2002) and ovine (Barber *et al.*, 2003) species. PII expression is ubiquitous including elevated expression in the mammary gland during lactation. PI is preferentially used in adipose tissue and liver under lipogenic conditions and in lactating

bovine mammary gland. PI generate ~30% of ACCα-encoded transcripts (Mao *et al*., 2001), while in ovine mammary gland its contribution is low (2%; Molenaar *et al*., 2003). The reason for this difference of promoter usage between the bovine and the ovine is unknown.

Fatty Acid Synthase (FAS)

Synthesis of medium-chain FA (C6-C16) in mammary gland during lactation occurs *via* universal FAS, which comprises 6 enzyme activity domains on a single protein of 2513 amino acids in ruminants. Functional FAS exists as an homodimer in a "head to tail" orientation (Wakil, 1989). In contrast to rodent mammary gland and duck uropygial gland, ruminant FAS synthesises medium-chain FA without the implication of a thioesterase II (Barber *et al*., 1997). Furthermore, in addition to being able to load acetyl-CoA, malonyl- and butyryl-CoAs, ruminant FAS contains a loading acyltransferase whose substrate specificity extends to up to 12 carbon atoms in the acyl chain, with the result that it is capable of loading and also releasing these medium-chain FA (Knudsen and Grunnet, 1982). This specificity of medium-chain FA synthesis is intrinsic to the lactating ruminant mammary gland, as the product of FAS in other ruminant tissues is predominantly C16:0, like in non-ruminant tissues (Christie, 1979). This enzyme is synthesised from long mRNA ranging in size from 8.4 to 9.3 kb depending on the species. In all studied human tissues (Jayakumar *et al*., 1995), in bovine mammary gland (Beswick and Kennelly, 1998), and in ovine (Bonnet *et al*., 1998) and porcine (Ding *et al*., 2000) adipose tissues, only one transcript has been detected by Northern-blot. However, in rat adipose tissue (Guichard *et al*., 1992) and mammary gland (Schweizer *et al*., 1989) two mRNA, generated by the use of two alternative polyadenylation signals, have been detected. To date the ruminant gene is cloned in bovine (GenBank accession number: AF285607) and the caprine cDNA was recently characterised (Leroux *et al*., 2005).

Stearoyl-CoA Desaturase (SCD)

SCD is a 359 amino acid protein located in the endoplasmic reticulum that introduces a Δ-9 bond in conformation *cis* on acyl-CoA from C14 to C19. In rodents, SCD relies on different genes whose expression and regulation by polyunsaturated FA (PUFA) are tissue-specific (Ntambi, 1999). By contrast, in ruminants there is only one SCD gene (Bernard *et al*., 2001), which generates one transcript of 5 kb in size which was characterised in sheep (Ward *et al*., 1998), cows (Chung *et al*., 2000) and goat (Bernard *et al*., 2001). In goat, as observed for human (Zhang *et al*., 1999), rat (Mihara, 1990) and mouse (Kaestner *et al*., 1989; Ntambi *et al*., 1988), the 3'-UTR sequence derives from a single exon and is unusually long (3.8 kb). Moreover, the caprine 3'-UTR is characterised by the presence of several AU-rich elements, considered as mRNA destabilisation sequences, and presents a genetic polymorphism consisting in the presence or absence of a triplet nucleotide (TGT) in position 3178-3180 (Bernard *et al*., 2001 and GenBank accession number AF325499). The known polymorphism of the caprine SCD gene has been recently enlarged by the detection of a single nucleotide substitution (G to T) in exon 5, at position 931 of the cDNA sequence, without amino acid change (Yahyaoui *et al*., 2003). Furthermore, Bernard *et al.* (unpublished) detected a polymorphism site consisting of a single nucleotide substitution (G to A) in exon 3, at position 521 of the cDNA sequence leading to an amino acid change (Val to Met). SCD mRNA in ovine (Ward *et al*., 1998) and activity in bovine (Kinsella, 1970) increased in the mammary gland immediately after parturition. In lactating goats, SCD gene is highly expressed

in the mammary gland and in subcutaneous adipose tissue, compared to perirenal adipose tissue (Bernard *et al*., 2005a). SCD in the lactating mammary gland is of considerable importance as this enzyme catalyses the Δ-9 desaturation of palmitoyl-CoA and stearoyl-CoA converting these to palmitoleoyl-CoA and oleyl-CoA (Enoch *et al*., 1976). SCD is also involved in the endogenous synthesis of *cis*-9, *trans*-11 (Corl *et al*., 2001; Griinari *et al*., 2000) and *trans*-7, *cis*-9 (Corl *et al*., 2002) CLA isomers in bovine mammary gland.

Genes of esterification of FA to glycerol

In ruminants, the distribution of FA on the glycerol backbone shows that a high proportion (56-62%) of FA esterified at positions *sn*-1 and *sn*-2 are medium- and long-chain saturated FA (C10:0 to C18:0), with C16:0 equally distributed among *sn*-1 and *sn*-2, C8:0, C10:0, C12:0 and C14:0 more located at *sn*-2, and C18:0 more located in *sn*-1 (Jensen, 2002). A high proportion of FA esterified at position *sn*-3 are short-chain FA (C4:0-C8:0; 44% on a molar basis) and oleic acid (27%) (Jensen, 2002). This non random distribution of FA on the 3 positions of the glycerol backbone determines functional and nutritional attributes of the milk triglycerides (German *et al*., 1997). Indeed, in humans, due to the specificity of pancreatic lipase for the *sn*-1 and *sn*-3 positions of the triglyceride molecule, the FA present at position *sn*-2 are preferentially absorbed because they remain in the monoacyl glycerol form (Small, 1991). Therefore the putative regulation of substrate specificity of the enzymes of FA esterification should be of major importance. Glycerol-3 phosphate acyl transferase (GPAT) catalyses the esterification of glycerol-3-phosphate in the *sn*-1 position, the first step in triglyceride biosynthesis. Two isoforms of GPAT have been detected in mammals, a mitochondrial and an endoplasmic reticulum isoform, which can be distinguished by their differential sensitivity to sulfhydryl group modifying agent N-ethylmalmeimide (NEM): the mitochondrial isoform is resistant to NEM, whereas the endoplasmic reticulum isoform is sensitive to NEM (Coleman and Lee, 2004) .

Regarding the second step of triglyceride synthesis, in accordance with the observed high proportion of medium- and long-chain saturated FA at position *sn*-2 with palmitate being the major FA (representing 43% of the total palmitate found in triacylglycerol), AGPAT (or Lysophosphatidic acid acyltransferase, LPAAT) has a greater affinity for saturated fatty acyl-CoA (Mistry and Medrano, 2002) in the order C16 > C14 > C12 > C10 > C8 (Marshall and Knudsen, 1977). Moreover, substrate availability in the bovine and ovine mammary gland is also a factor for the *sn*-2 position FA composition which means that it may be manipulated to some extent by nutritional factors, in interaction with the aforementioned substrate affinity. Bovine and ovine AGPAT genes were recently characterized, cloned and located on bovine chromosome 23 (Mistry and Medrano, 2002). Bovine and ovine genes encode proteins of 287 amino acids, differing by one animo acid residue.

DGAT is the only enzyme that is specific to triacylglycerol synthesis and, therefore, may be the rate-limiting step in triacylglycerol synthesis (Mayorek *et al*., 1989). The protein is known to be located on the endoplasmic reticulum membrane. Little is known about the regulation of DGAT expression whereas its gene has been particularly well studied in ruminant due to its genetic variability. A QTL for milk fat content has been detected in the centromeric region of cattle chromosome 14 and DGAT1 gene, located in the same region (Grisart *et al*., 2002), was

proposed as a positional and functional candidate for this trait (Grisart *et al*., 2002 ;Winter *et al*., 2002). Both studies, found a nonconservative substitution of lysine by alanine (K232A) in DGAT1 caused by AA to GC dinucleotide substitution at position 10434, in exon 8. Recently, Grisart *et al.* (2004) demonstrated *in vitro* that the K allele is associated with increased activity of the enzyme in agreement with its positive link with bovine milk fat percentage.

Effect of dietary factors on lipogenic genes expression in the mammary gland

Regarding nutritional regulation, both macronutrients (carbohydrates, FA, protein and/or amino acids) and micronutrients (trace metals, vitamins) participate, in concert with endocrine factors in the regulation of gene expression in response to dietary changes (Clarke and Abraham, 1992). Specific transcription factors can bind the promoter region and thus regulate the transcription of the relevant gene. The regulation of gene expression could also occur during conversion of the primary transcript to mRNA (including splicing, polyadenylation, etc). The stability of the mRNA as well as their translation (accessibility, initiation, etc) are other steps involved in gene expression regulation. Finally, turn-over and activation of the enzymatic protein, could be the last key-step of regulation.

So the response of gene expression to nutrients involves control of events that could occur at each of these levels: transcription, post-transcription, translation and post-translation but it is often unclear whether or not either the regulatory factors are the dietary components themselves, or their metabolites or hormonal changes produced in response to the nutritional changes. In addition, it is difficult to conclude on the level of regulation involved since, for a given gene, measurements of the relevant mRNA, of the enzyme protein content and activity are generally not made simultaneously, as it is the case in the studies reported in this review.

In ruminants, only limited data are available on the nutritional regulation of mammary lipogenic gene expression either *in vivo* or *in vitro*. Few *in vivo* trials have been carried out in mid-lactation cows and goats.

In cows, data are mainly (3 out of the 4 studies) from studies with milk fat depressing (MFD) diets which consisted of:

- **trial 1**: a high level of concentrate (mainly composed of 76% cracked corn, 19% heat-treated soybean meal, 1% sunflower oil) with a forage (alfalfa hay) to concentrate ratio of 16/84, compared to a control diet with a high forage (composed of 83% corn silage and 17% alfalfa hay) content and a forage to concentrate ratio of 53/47 (Peterson *et al*., 2003; 3 cows).
- **trial 2**: a high level of concentrate (composed of 52% ground corn, 15% soybean meal) with a forage (corn silage) to concentrate ratio of 25/70, supplemented with 5% of soybean oil, compared to a high forage (76% corn silage and 24% alfalfa haylage) content with a forage to concentrate (composed mainly of 57% ground corn and 34% soybean meal) ratio of 60/40 (Piperova *et al*., 2000; 10 cows).
- **trial 3**: a diet based on grass silage (19%), corn silage (19%) and rolled barley (44%) supplemented, or not, with 2.7% of fish oil either "protected" or non-protected (Ahnadi *et al*., 2002; 16 cows).

- **trial 4**: a diet based on grass (27%), corn silage (30%) and rolled barley (22%) supplemented with 3.3% unprotected canola seeds plus 1.5% canola meal, or 4.8% formaldehyde-protected canola seeds, compared to the same diet with 4.8% of canola meal, which represents for the 2 treatment diets a supply of 0.7 and 0.6% of extra-lipids compared to the control diet, respectively (Delbecchi *et al*., 2001; 6 cows).

In goats, the data were obtained from supplementations with dietary lipids varying in their nature, form of presentation and dose. The dietary treatments consisted of:

- **trial 5**: a 54% hay diet supplemented, or not, with 3.6% of lipids from oleic sunflower oil or formaldehyde-treated linseeds, with the concentrate fraction composed of 40% rolled barley, 17% dehydrated sugar beet pulp, 10% pelleted dehydrated lucerne and 17% soybean meal (Bernard *et al*., 2005b; 14 goats).
- **trial 6**: two diets with 47% of either hay (13 goats) or corn silage (14 goats), supplemented, or not, with 5.8% of either linseed or linoleic sunflower oil, with the concentrate fraction composed of 24-24% rolled barley, 39-34% dehydrated sugar beet pulp, 12-0% pelleted dehydrated lucerne and 25-42% soybean meal, respectively (Bernard *et al*., unpublished; 27 goats).

Contrary to what is generally observed in dairy cows (Bauman and Griinari, 2003; Chilliard and Ferlay, 2004), dietary lipid supplementation in the present goat studies always increased the milk fat content and did not increase (or only slightly increased) milk yield, in accordance with previously published goat studies (Chilliard *et al*., 2003a). The data base constituted from these cow and goat studies allowed us to evaluate how dietary factors changed metabolic pathways and gene expression, in interaction with animal species peculiarities.

Regulation of mammary lipoprotein lipase

In cows, studies on MFD diets reported either no change in the abundance of mammary LPL mRNA (trial 3; Ahnadi *et al*., 2002) or a tendency to decrease (trial 1; Peterson *et al*., 2003). Moreover, again with a MFD diet, no change in the abundance of FABP has been observed (Peterson *et al*., 2003). Nevertheless, in these 2 studies milk long chain FA (> C16) secretion (g/d) was significantly decreased, by 43% after fish oil supplementation (trial 3) and by 28% with a high-concentrate diet (trial 1). Elsewhere, in goats fed a hay based diet supplemented with oleic sunflower oil (trial 5; Bernard *et al*., 2005b), no significant effect was observed on LPL activity while mammary LPL mRNA content was increased, simultaneously to a large increase (83%) in long chain FA (C18) secretion. In goats fed hay or corn silage supplemented with either linseed or sunflower oil (trial 6; Bernard *et al*., unpublished), we observed a large increase in the secretion of milk long-chain FA (>100%) without effect on mammary LPL activity and mRNA except when corn silage was supplemented with sunflower oil for which an increase in LPL activity was observed. Similarly, in late-lactating goats fed alfalfa hay-based diet, supplementation with 3.8% lipids from soybeans had no effect on mammary LPL mRNA abundance while the secretion of long-chain FA (C18) in milk was increased by 58% (Bernard *et al*., 2005a). Thus, most of the results in ruminants are not in agreement with studies in rodents (Del Prado *et al*., 1999), in which a very high dietary lipid intake (20%) enhanced both mammary gland LPL activity and lipid uptake.

The results in ruminants suggest that the total LPL activity measured *in vitro* in optimal conditions is generally not the limiting factor in the uptake of long-chain FA by the mammary gland. Indeed the availability of plasma triglyceride FA could play an important role (Gagliostro *et al*., 1991), as well as the location of LPL (capillary endothelial cells, MEC or depleted adipocytes). These results are also in accordance with those from previous studies in lactating goats, demonstrating that mammary utilisation of plasma triglyceride and NEFA is related to their arterial concentration (see earlier section). Moreover, Chilliard *et al.* (2003b) observed the existence of positive correlations (Figure 2) between milk stearic acid percentage and milk fat content, related both to the response to dietary lipids and to individual variations within each dietary treatment. This suggests that a significant availability and uptake of this FA is an important factor for milk fat secretion in goats, as in cows (Loor *et al*., 2005a).

In other respects, a large decrease in goat milk LPL activity (and post-milking lipolysis) is generally observed when lipid supplements are added to the diet. It was hypothesised that milk LPL decreases when more mammary LPL enzyme is directed towards the basal membrane of MEC and the capillary lumen to allow increased uptake of blood triglycerides arising from digestive absorption. Thus, less enzyme would be transported from the MEC to the milk, according to the model for LPL transport in the mammary gland proposed by Jensen *et al.* (1991) in mice and Chilliard *et al.* (2003a) in goats.

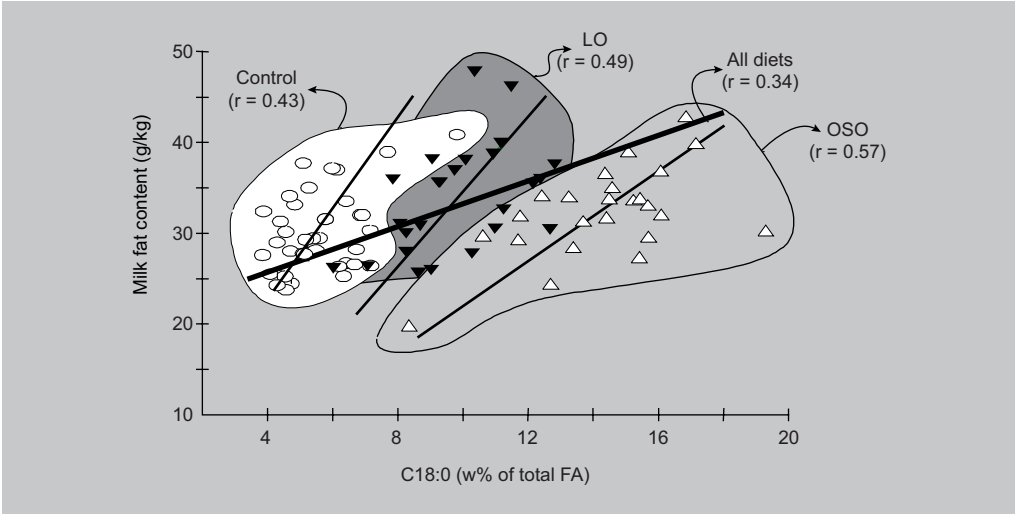

Figure 2. Relation between milk fat content and stearic acid percentage in 84 goats fed 7 diets differing in either forages: fresh raygrass + raygrass hay (.26 kg/d) vs. raygrass hay vs. alfalfa hay, or lipid intakes (0 g/d, control (n=36) vs. 130 g/d, i.e. 5-6% of diet DM, of either linseed oil, LO (n=24) or high oleic sunflower oil, OSO (n=24)) (Source: adapted from Chilliard et al.*, 2003b and unpublished).*

Regulation of genes of *de novo* lipid synthesis (ACC and FAS)

In cows, it was shown (trial 2; Piperova *et al*., 2000) that a MFD diet induced reductions in ACC mRNA abundance and activity, and in FAS activity in mammary tissue, along with a dramatic decrease of 60% in C4-C16 FA secretion (g/d). Elsewhere, Ahnadi *et al.* (2002; trial 3) using diets supplemented with fish oil observed a decrease in mammary ACC and FAS mRNA levels together with a decrease of 38% in C4-C16 FA secretion. Furthermore, the reductions of milk fat secretion (27% decrease) and of C4-C16 (30% decrease) observed in cows with a high-concentrate diet (trial 1; Peterson *et al*., 2003) were accompanied by a reduction of mammary mRNA abundance of numerous genes involved in the various pathways of milk fat synthesis, including those of ACC and FAS, but without effect on milk *K*-casein mRNA.

In goat studies (trials 5-6; Bernard *et al*., 2005b; unpublished), a decrease of 15 to 22% in milk C4-C16 FA secretion (g/d) was observed after supplementation of hay or corn silage diets with vegetable lipids, that was not accompanied by any significant variation of ACC and FAS mRNA levels or activities. Nevertheless, from individual values obtained by biopsies from goats fed hay or corn silage diets supplemented with either 5.8% of linseed or sunflower oil (trial 6; Bernard *et al*., unpublished), a positive relation (r = + 0.62) was observed between the variation of ACC mRNA abundance and the C4–C16 secretion response to lipid supplementation (Figure 3a). From results of cow and goat studies, ACC mRNA abundance and C4-C16 secretion responses to dietary treatment were positively related (r = + 0.66; Figure 3b). These results suggest that the variation of ACC mRNA abundance plays a role in the response of milk short- and medium-chain FA to dietary manipulation, especially addition of lipids to the diet.

Some of the differences observed between cows (Ahnadi *et al*., 2002; Piperova *et al*., 2000) and goats (Bernard *et al*., 2005b) in the magnitude of responses of ACC (Figure 3b) and FAS mRNA and/or activity to dietary PUFA supplementation may be explained by the level of starchy concentrate in the diet, the nature, FA composition (fish oil vs vegetable oil) and presentation (seeds vs free oil) of the lipid supplements, as well as by species differences. This last point is supported by the fact that lipid supplementation in goats always induces an increase in milk fat content and secretion whereas it generally decreases it in cows (see earlier section).

From the available *in vivo* studies where both ACC and FAS mRNA levels and activities in the mammary gland were measured (our goat data and Piperova *et al.* (2000) for ACC in cow), a positive relation between the variation of mRNA and activities in response to dietary treatment was observed for ACC (r = + 0.91) and FAS (r = + 0.89) (Figure 4) suggesting a regulation at a transcriptional level, at least. This level of regulation for ACC and FAS is in accordance with data obtained in rat adipose tissue (Girard *et al*., 1997; Lopez-Casillas *et al*., 1991) and liver (Girard *et al*., 1994; Lopez-Casillas *et al*., 1991) in response to nutritional factors. For ACC, this level of regulation occurs in addition to the well documented post-translational regulation (see earlier section).

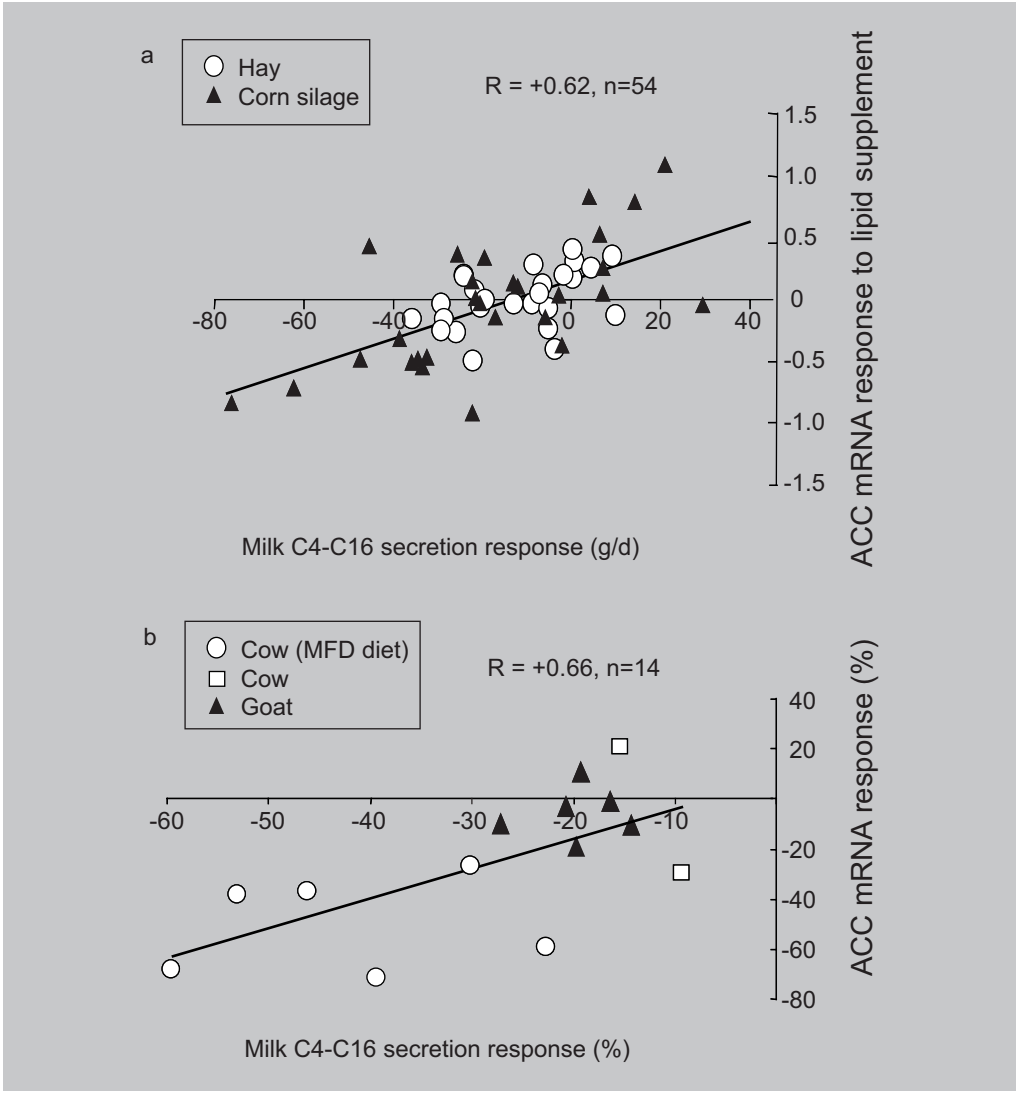

Figure 3. Relationships between milk short- and medium-chain fatty acid and mammary acetyl-CoA carboxylase (ACC) mRNA level responses to different dietary treatments.

a. In 27 goats (trial 6, see text). Results from 54 individual responses (lipid supplements – control) from 81 mammary biopsies in two 3x3 latin squares. Lipid supplements were linseed oil or sunflower oil (Source: Bernard et al.*, unpublished).*

b. In goat (trials 5-6; n=41; Bernard et al.*, 2005b; Bernard* et al.*, unpublished) and cow (Baumgard* et al.*, 2002 and trials 1-4; n=39; Ahnadi* et al.*, 2002; Delbecchi* et al.*, 2001; Peterson* et al.*, 2003; Piperova* et al.*, 2000). Results are means per group. Dietary treatments are described in the text and consisted of either lipid supplementation in goat (▲) and cow (□), or milk fat depressing (MFD) diets or post-ruminal trans-10, cis-12 CLA infusion (13.6 g/d) in cows (○).*

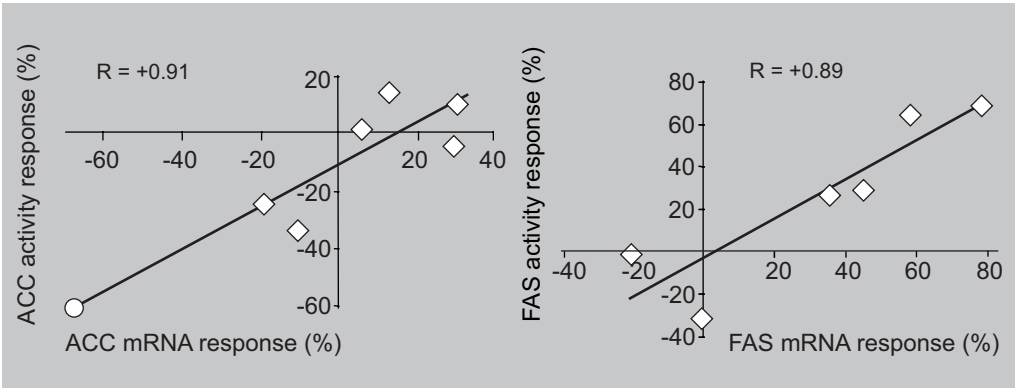

Figure 4. Relationships between mammary acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) mRNA and activity responses to dietary lipids in 41 goats (◇) fed either hay- or corn silage-based diets and 10 cows (○) fed high-concentrate diet. The lipid supplements, in the goat studies, were either 3.6% of lipids from oleic sunflower oil or formaldehyde-treated linseed (trial 5; Bernard et al.*, 2005b), or 5.8% lipids from linseed oil or sunflower oil (trial 6; Bernard* et al.*, unpublished), and in the cow study, 5% of soybean oil (trial 2; Piperova* et al.*, 2000).*

Regulation of stearoyl-CoA desaturase

In rodents, nutritional regulation of SCD activity mainly occurs in the liver and has been studied extensively (Ntambi, 1999). In the ruminant lactating mammary gland, only few studies investigated its nutritional regulation at the mRNA abundance and/or protein activity level.
In cows, feeding 2.7% of fish oil supplement (rich in long-chain n-3 FA) (trial 3; Ahnadi *et al.*, 2002) decreased mammary SCD mRNA abundance.

In goats fed hay-based diets supplemented with lipids, a decrease in mammary SCD mRNA abundance was observed with formaldehyde treated linseed while a decrease in enzyme activity was observed with oleic sunflower oil, linseed oil and sunflower oil, whereas with a corn silage diet no effect of lipid supplementation on SCD mRNA or activity was observed (trials 5-6; Bernard *et al.*, 2005b). Similarly, in late-lactating goats fed an alfalfa hay-based diet, supplementation with 3.8% lipids from soybeans had no effect on mammary SCD mRNA (Bernard *et al.*, 2005a). All together goat results suggest (i) an interaction between the basal diet and the dietary lipids used and (ii) a negative transcriptional or post-transcriptional regulation by dietary PUFA and/or by their ruminal biohydrogenation products.

As SCD activity measurement needs fresh materials and is laborious (Legrand *et al.*, 1997), SCD activity has often been estimated by the milk ratios for the pairs of FA that represent a product/substrate relationships for SCD (*cis*-9 C14:1/C14:0, *cis*-9 C16:1/C16:0, *cis*-9 C18:1/C18:0, *cis*-9 *trans*-11CLA /*trans*-11 C18:1; Bauman *et al.*, 2001). In our goat studies the 4 FA pair ratios representing a proxy for SCD activity were more or less related to the SCD activity itself, across 6 dietary groups (Figure 5). The response to dietary lipids (4 comparisons) of the milk myristoleic acid to myristic acid (*cis*-9 C14:1/C14:0) ratio gave the best proxy for the response of mammary

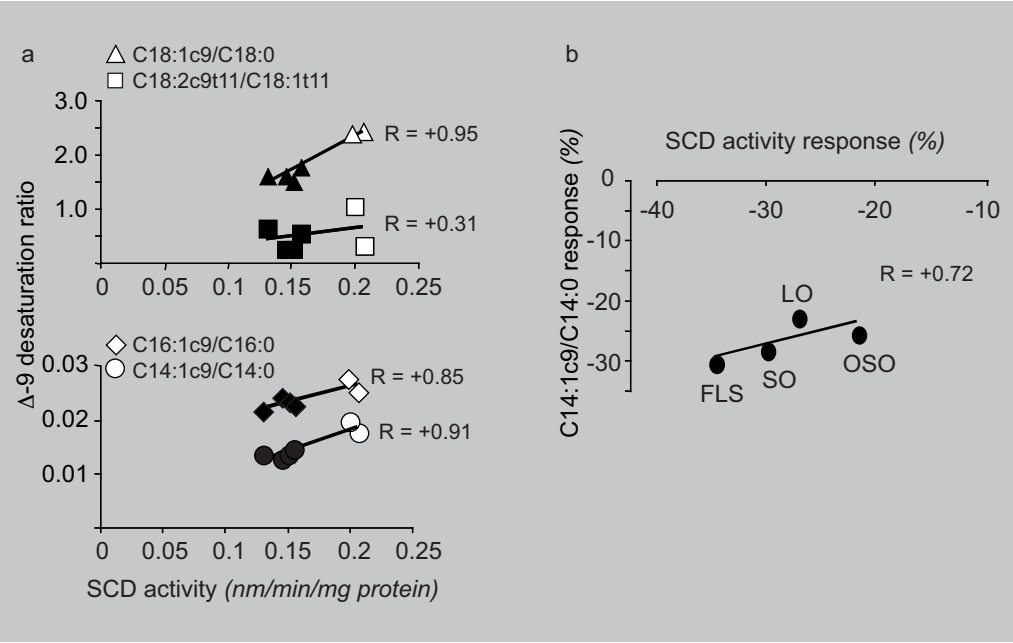

Figure 5. Relationships between milk FA desaturation ratios and stearoyl-CoA desaturase (SCD) activity in 27 goats fed hay-based diet supplemented, or not, with lipids. The lipid supplements were either 3.6% of lipids from oleic sunflower oil (OSO) or formaldehyde-treated linseed (FLS) (trial 5; Bernard et al.*, 2005b), or 5.8% lipids from linseed oil (LO) or sunflower oil (SO) (trial 6; Bernard* et al.*, unpublished).*
a. Results are means of the 4 lipid-supplemented groups (solid symbols) and the 2 control groups (open symbols).
b. Relationship between the responses (%) of SCD activity and milk cis-9 C14:1/C14:0 to the 4 lipid supplements.

SCD activity. Myristic acid originates almost exclusively from *de novo* synthesis within the mammary gland (because C14:0 is poorly represented in ruminant feedstuffs, including lipid supplements) and, as a consequence, almost all the myristoleic acid present in the milk is likely to be synthesised in the mammary gland by SCD. Conversely, as variable proportions of palmitoleic, palmitic, oleic, stearic, vaccenic and rumenic acids come from absorption from the digestive tract and/or mobilisation of body fat reserves, these ratios are less indicative of SCD activity. In addition, all the 4 FA pair ratios could be influenced by other factors than SCD activity, such as: (i) differential accuracy in the quantification of the *cis*-9 isomers in milk, (ii) differential uptake of the different FA by the mammary gland and (iii) differential turnover and use of these different FA by the mammary tissue itself.

Regulation of acyltransferases

Regarding genes encoding enzymes involved in FA esterification, mammary mRNA abundance of the GPAT and AGPAT was reduced in cows fed a high concentrate diet which led to a 27%

reduction of milk fat yield (Peterson *et al*., 2003). Data on these genes are not yet available in goats.

Molecular mechanisms involved in nutritional regulation of gene expression

A central regulatory role for specific fatty acids

In vivo results in feeding trials

A role for long-chain saturated FA and/or PUFA in decreasing mammary *de novo* FA synthesis has been suggested from earlier in vivo studies (Chilliard *et al*., 1991). However, a central role for specific *trans*-FA as potent inhibitors of mammary lipid synthesis has been proposed recently, from results observed in specific dietary conditions that induce a dramatic MFD (Bauman and Griinari, 2003). Similarly, in rats fed a diet containing a mixture of *trans*-isomers, an impairment of mammary lipid synthesis has been observed (Assumpcao *et al*., 2002).

The "low-milk fat syndrome" in cows seems indeed to be mainly due to the synthesis within the rumen of specific PUFA biohydrogenation products. The diets that induce MFD belong to three groups: (i) diets rich in readily digestible carbohydrates and poor in fibrous components, without addition of lipid supplements (*e.g.* high grain/low forage diets; Peterson *et al*., 2003), but containing a minimal amount of PUFA in dietary feedstuffs (Griinari *et al*., 1998), (ii) low fibre diets associated with supplemental PUFA of plant origin (Piperova *et al*., 2000), and (iii) dietary supplements of marine oils (fish oils, fish meals, oils from marine mammals and/or algae) which induce MFD whatever the level of starch or fibre in the diet (Ahnadi *et al*., 2002; Chilliard *et al*., 2001). A number of theories have been proposed in the past to explain diet-induced MFD, with alteration in ruminal fermentations as the starting point for all these theories (reviews by Bauman and Griinari, 2001, 2003). One is that the alteration results in rumen production of acetate and butyrate which is too low to support milk fat synthesis. Another is that increased production of propionate in the rumen enhances the hepatic rate of gluconeogenesis, and increases levels of circulating glucose and insulin and adipose tissue lipogenesis, thus inducing a shortage of nutrients available for the mammary gland. Another theory, which is now prevailing, was first proposed by Davis and Brown (1970), and suggests that mammary fat synthesis is inhibited by specific *trans*-FA which result from alterations in rumen PUFA biohydrogenation.

In cows, *in vivo* studies support the *trans*-FA hypothesis because an increase in the *trans*-C18:1 percentage of milk fat is observed for a wide range of MFD diets (review by Bauman and Griinari, 2003). An important development of this theory was the discovery by Griinari *et al.* (1998) that MFD was associated with a specific increase in *trans*-10 C18:1 rather than *trans*-C18:1 isomers in general, which was then confirmed in several studies in cows from which a curvilinear response-curve (Figure 6) was evidenced (Loor *et al*., 2005a). A curvilinear relationship between the decrease in milk fat percentage and small increases in milk *trans*-10, *cis*-12 CLA (Bauman and Griinari, 2003) was also observed in some studies, but not all. For example, the reduction of milk fat (27% decrease) and of C4-C16 (30% decrease) secretion observed in cows with a high-concentrate diet (trial 1; Peterson *et al*., 2003) was accompanied by a small but significant increase in milk fat *trans*-10, *cis*-12 CLA secretion (+ 0.5 g/d).

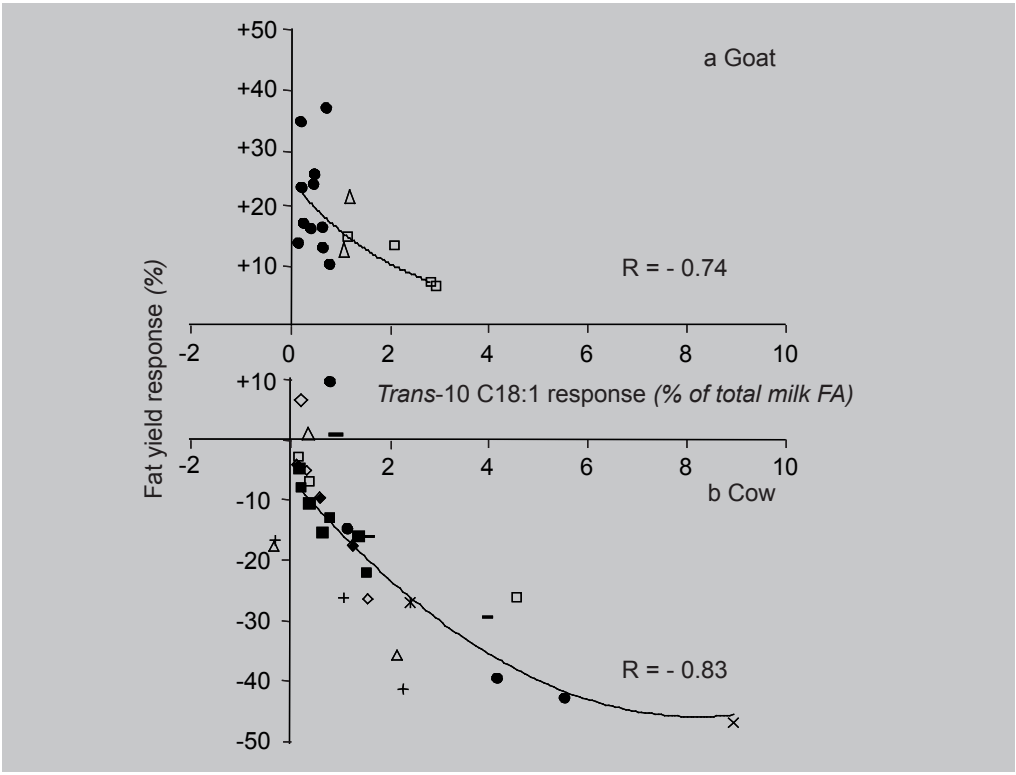

Figure 6. Relationships between milk trans-10 C18:1 and fat yield responses.
a. Goat studies: data from 17 lipid-supplemented groups, compared to 8 control groups (253 goats). The forages were either hay (●), fresh grass (△) or corn silage (□). The lipids supplements were either sunflower oil, oleic sunflower oil, linseed oil or extruded linseeds (4-6% lipids in diet DM) (Source: adapted from Chilliard et al.*, 2003a; Chilliard and Ferlay, 2004; Bernard* et al.*, unpublished (trial 6)).*
b. Cow studies: data from 31 lipid- or concentrate-supplemented groups, compared to control groups (13 studies reviewed by Loor et al.*, 2005a).*

Regarding *trans*-10 C18:1, this isomer is probably formed in the rumen from the reduction of *trans*-10, *cis*-12 CLA in an alternative pathway for biohydrogenation of linoleic acid, which increases when rumen pH decreases. This "*trans*-10 pathway" seems indeed to increase with diets rich in concentrate and/or corn silage, and to occur after a 1-2 weeks period of latency following dietary PUFA supplementation, during which an earlier but transient increase in the "*trans*–11 pathway" occurred (Chilliard and Ferlay, 2004; Roy *et al.*, 2004).

It is noteworthy that in goats as in cows, a negative and curvilinear relationship is observed between the responses of milk *trans*-10 C18:1 percentage and milk fat yield (Figure 6), despite the fact that the fat yield response was always positive in goats, but always negative or null in cows. In goats, the greatest increases in *trans*-10 C18:1 were observed with either corn silage or fresh grass diets (Figure 6) and corresponded to the lowest increases in milk fat yield. Furthermore, we did

not observe any significant increases in goat milk *trans*-10, *cis*-12 CLA in the 17 diet comparisons in Figure 6. These similarities and differences among ruminants suggest a species specificity of FA ruminal and/or mammary metabolism. The positive effect of lipid supplementation on goat milk fat yield could be due to the mammary sensitivity to increased availability of stearic acid arising from dietary PUFA biohydrogenation in the rumen. However, this factor seems to be more important to explain within-group individual variability, than differences between dietary groups (Figure 2).

Regarding SCD gene expression, little is known yet concerning PUFA effects on its regulation, in ruminants. Conversely, in rodents, the down-regulation of SCD gene expression by (n-6) and (n-3) PUFA has been comprehensively investigated in liver and AT (Ntambi, 1999) and to a lower extent in the lactating mammary gland (Lin *et al*., 2004; Singh *et al*., 2004). Thus Singh *et al.* (2004) demonstrated a negative effect of olive oil or safflower oil when fed to lactating mice on both SCD mRNA and activity in the mammary gland and SCD mRNA in the liver. Still in lactating mice, Lin *et al.* (2004) showed a decrease in mammary SCD, ACC and FAS mRNA abundance and SCD activity by the addition of either *trans*-11 C18:1, *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA, whereas these FA treatments had no effect on these 3 mRNA in the liver. However, in the liver, both *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA had a negative effect on SCD activity. These results on lactating mice indicate a tissue-specific regulation of lipogenic gene expression by *trans*-FA, and outline the possibility to manipulate mammary SCD gene expression by nutrition. However, the interaction of the physiological state on these regulations needs to be clarified.

In goats, part of the observed negative effect of oleic sunflower oil, sunflower oil and linseed oil when added to hay diet (trials 5-6) on SCD activity (Figure 5), and of formaldehyde treated linseed on SCD mRNA, might be attributed to dietary *cis*-9 C18:1, C18:2 (n-6) and/or C18:3 (n-3) escaping from the rumen and/or to *trans*-isomers formed during ruminal metabolism of these 3 FA (Chilliard *et al*., 2003c; Ferlay *et al*., 2003; Rouel *et al*., 2004). Indeed, besides PUFA biohydrogenation processes, oleic acid could be isomerised in several *trans*-C18:1 isomers, including *trans*-10, in microbial cultures from bovine rumen (Mosley *et al*., 2002) in agreement with the increase of several *trans*-C18:1 isomers observed in milk from goats fed oleic sunflower oil (Bernard *et al*., 2005b; Ferlay *et al*., 2003). However, data in Figure 5b suggest that α-linolenic acid from formaldehyde treated linseeds should be more efficient than dietary oleic acid-rich oil to decrease SCD activity.

Duodenal infusion of trans-10, cis-12 CLA

The hypothesis that *trans*-10, *cis*-12 CLA could be a potent inhibitor of milk fat synthesis was initiated by duodenal infusion trials of pure CLA isomers that demonstrated that *trans*-10, *cis*-12 CLA inhibited milk fat synthesis in dairy cows, whereas the *cis*-9, *trans*-11 CLA isomer had no effect (Baumgard *et al*., 2000; Loor and Herbein, 2003). Furthermore, two other CLA isomers, *trans*-8, *cis*-10 CLA and *cis*-11, *trans*-13 CLA, naturally present at very low concentrations in the rumen and milk, did not inhibit milk fat yield when infused at high level (4 g/d) (Perfield *et al*., 2004). However, Baumgard *et al.* (2002) showed that the severe reduction (48%) in milk fat yield due to the infusion of a high dose (13.6 g/d) of *trans*-10, *cis*-12 CLA (*vs* the < 0.1 g/d flowing at the duodenum with a MFD diet rich in concentrate and supplemented with linseed oil; Loor *et al*.,

2004a, 2005a) was accompanied by a dramatic reduction (more than 35%) of mRNA abundance of enzymes involved in mammary uptake and intracellular trafficking of FA (LPL and FABP), *de novo* FA synthesis (ACC and FAS), desaturation (SCD), and esterification (GPAT and AGPAT). Nevertheless, the levels of *trans*-10, *cis*-12 CLA in the rumen, duodenal fluid or milk always remained very low (in absence of duodenal infusion) compared to the levels reached by the *trans*-10 C18:1 isomer (ratio between *trans*-10, *cis*-12 CLA and *trans*-10 C18:1 of ~0.01; Bauman and Griinari, 2003; Loor *et al*., 2004a, 2004b, 2005a; Piperova *et al*., 2000). Furthermore, little or only traces of *trans*-10, *cis*-12 CLA were detected during MFD in cows fed marine oil, whereas substantial increases in *trans*-10 C18:1 were observed (Loor *et al*., 2005b; Offer *et al*., 2001).

Because of the lack of pure material, it was not possible up to now to demonstrate a direct effect of *trans*-10 C18:1 on milk fat synthesis, whereas there is a clearly established potent inhibitory effect of *trans*-10, *cis*-12 CLA when post-ruminally infused in dairy cows (Bauman and Griinari, 2003). Furthermore, these authors suggested that rumen environment alterations which result in the formation of *trans*-10 C18:1 and of *trans*-10, *cis*-12 CLA would likely result also in other biohydrogenation intermediates that could inhibit milk fat synthesis. So several other rumen-derived FA were proposed by Loor *et al.* (2005a) as potential inhibitors of cow milk fat synthesis: *cis*-11 C18:1, *cis*-15 C18:1, *trans*-6+7+8 C18:1, *trans*-9 C18:1, *trans*-11 C18:1, *trans*-12 C18:1 and *cis*-9, *trans*-13 C18:2, *cis*-9, *trans*-12 C18:2, *trans*-11, *cis*-13 CLA and *trans*-11, *cis*-15 C18:2. Nevertheless, here again, most of these isomers increased in goats supplemented with dietary PUFA whereas no MFD was observed (Chilliard and Ferlay, 2004).

In vitro results

Several years ago, *in vitro* studies on dispersed bovine MEC (Hansen *et al*., 1986; Hansen and Knudsen, 1987) showed that the addition of C18:0, *cis*-9 C18:1 or C18:2 (n-6) inhibited *de novo* synthesis of FA with 16 carbons or less, except C4. Moreover, 35 years ago, Bickerstaffe and Annison (1970) observed negative *in vitro* effects of oleic, linoleic and linolenic acids on goat mammary SCD activity, which are partly in accordance with our *in vivo* results on goat (see earlier secion). Recently, looking at the effects of specific FA in bovine mammary cell line cultures, Jayan and Herbein (2000) showed that, compared to stearic acid, *trans*-11 C18:1, and to a lower extent *cis*-9 C18:1, reduced ACC and FAS activities. Regarding the SCD, addition of *trans*-11 C18:1 *in vitro* increased its mRNA abundance in bovine MEC (Matitashvili and Bauman, 2000) and activity in bovine MAC-T cell line (Jayan and Herbein, 2000). The effect of other specific *trans*-C18:1 isomers, in particular of *trans*-10, on mammary SCD gene expression *in vitro* is still unknown.

Thus further research is needed to identify the more important inhibitors of fat synthesis either *in vivo* (i.e. post-ruminal infusion of exogenous FA) or *in vitro*, either in cows or in other ruminants, but this research is hampered by the lack of pure *trans*-C18:1 and C18:2 isomers, and also by the difficulty to obtain an *in vitro* functional model for lipid synthesis and secretion (Barber *et al*., 1997; Neville and Picciano, 1997).

Signalling pathways potentially mediating nutritional regulation of gene expression

In ruminants, little is known about the signalling mechanisms involved in the regulation of lipogenic gene expression whereas these mechanisms have been partially described in rodent liver and adipose tissue (Clarke, 2001). Nevertheless, because a coordinate down-regulation of several genes involved in milk fat synthesis has been observed in the bovine mammary gland in response to a post-ruminal infusion of *trans*-10, *cis*-12 CLA (Baumgard *et al*., 2002) and to a MFD diet (Peterson *et al*., 2003), it has been suggested that these genes may share a common regulatory mechanism. Clarke (2001) proposed that PUFA-regulation of gene transcription in rodents occurs via a ligand-mediated event, such as FA binding to, or regulating, a transcription factor. Sterol regulatory binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptors (PPARs) are the main candidates. In fact, the regulation of gene transcription by FA could be due to changes in either the activity or abundance of transcription factors. Whereas FA or cholesterol bind to nuclear receptors as PPAR (and LXR, HNF-4α), they do not bind to SREBP protein but induce changes in the nuclear abundance of this transcription factor.

SREBP-1

SREBP transcription factors belong to a family of transcription factors which regulate genes involved in cholesterol and FA synthesis. Currently three SREBP forms have been characterised in rodents, SREBP-1a, -1c and -2. The SREBP-1a form is mainly expressed in cultured cells and tissues with a high cell proliferation capacity. The SREBP-1c form is expressed in many organs (mainly adipose tissue, brain, muscle…) (Shimomura *et al*., 1997). Both forms derive from a single gene through the use of alternate promoters that give rise to different first exons (Brown and Goldstein, 1997). They play an important role in the induction of the transcription of genes involved in the synthesis of FA, triglycerides, and phospholipids. SREBP-2 derives from a different gene and is involved in the transcription of cholesterogenic enzymes.

SREBPs are synthesised from ~ 1150 amino acid precursor proteins that remain bound to the endoplasmic reticulum and nuclear envelope in the presence of sufficient sterol concentrations. Upon sterol deprivation, the precursor protein is escorted by SREBP cleavage-acting protein (SCAP) to the Golgi compartment where the SREBPs are processed by site-1 and site-2 proteases (S1P and S2P) to release the NH_2-terminal portion (Sakai *et al*., 1996). This NH_2-terminal portion, mature SREBP, then enters the nucleus and activates the transcription of genes implicated in cholesterol and FA synthesis, including LPL, ACC, FAS and SCD (Shimomura *et al*., 1998), by binding to sterol binding elements or to palindromic sequences called E-boxes within their promoter regions (Kim *et al*., 1995; Wang *et al*., 1993).

FA induce decreases in the nuclear abundance of SREBP-1. So far two mechanisms for this down-regulation have been described in rodents, either an inhibition of the proteolytic process of activation of SREBP-1 protein (under cholesterol dependence) or an inhibition of the transcription of the SREBP-1 gene. Recently, Barber *et al.* (2003) identified SREBP-1 in the ovine lactating mammary gland as a major regulator of *de novo* lipid synthesis through the activation of ACCα PIII, achieved together with NF-Y, USF-1 and USF-2 transcription factors. SREBP-1 binding motifs are also present in the proximal promoter of ACCα PII, which is up-regulated during lactation, indicating that SREBP-1 could play an important role in the co-ordinate regulation of

PII and PIII in mammary tissue (Barber *et al*., 2003). Elsewhere, Peterson *et al.* (2004) reported, in bovine MEC line (MAC-T), that addition of *trans*-10, *cis*-12 CLA had no effect on SREBP-1 mRNA or precursor protein content but reduced proteolytic activation of the precursor SREBP-1 with a subsequent reduction in transcriptional activation of the lipogenic genes ACC, FAS and SCD, explaining the inhibitory effect of this CLA isomer on lipid synthesis.

PPARs

PPARs define a subfamily of nuclear hormone receptors, regulating the transcription of genes involved in lipid metabolism, mainly in the transport of plasma triglycerides (through the regulation of the expression of apolipoprotein-AI and apolipoprotein-CIII), cellular FA uptake, peroxisomal and mitochondrial β-oxidation (Schoonjans *et al*., 1996a). PPARs are activated by binding diverse compounds (including FA and their metabolites, hypolipidemic drugs, steroids and thiazolidinediones) and then heterodimerise with the *cis*-9 retinoic acid receptor (RXR) to bind to specific response elements located in the promoter region of the target genes. Three PPAR subtypes α, β, and γ have been identified in vertebrates with specific physiological functions. PPARα, expressed mainly in liver as well as in heart, kidney, intestinal mucosa, and brown adipose tissue (tissues with high catabolic rates for FA), is involved in FA transport and β– and ω-oxidation.

PPARβ is abundantly and ubiquitously expressed and mainly found in heart, lung and kidney. PPARγ, most abundant in adipose tissue, stimulates adipocyte differentiation and lipogenesis of the mature adipocyte (Schoonjans *et al*., 1996b). PPARγ is also expressed in a number of epithelial tissues which are important in human breast, prostate and colon cancer, because it seems to favour less malignant phenotype cells (Sarraf *et al*., 1999). PPARγ gene generates two transcripts, designated PPARγ1 and γ2, resulting from differential mRNA splicing and promoter usage (Yeldandi *et al*., 2000), and leading to two protein isoforms with PPARγ2 containing 30 additional amino acid residues at the N terminal extremity compared to PPARγ1. Whereas many tissues express a low level of PPARγ1, PPARγ2 is expressed at very high level in adipose tissue (Rosen and Spiegelman, 2001). The peroxisome proliferators, including chemical molecules as fibrates, thiazolidinedione and natural molecules as PUFA and derivatives, activate the transcriptional activity of the PPAR genes. However, (n-3) and (n-6) PUFA and mainly their metabolites, such as eicosanoids and oxidised FA, are the major natural activators of PPARα (Clarke, 2001) while 15-deoxy-$\Delta^{12, 14}$-prostaglandin J2 is the activator of the PPARγ subtype (Rosen and Spiegelman, 2001). Moreover, *in vitro* studies on mature adipocytes revealed that *trans*-10, *cis*-12 CLA down-regulates PPARγ gene expression, suggesting that this CLA isomer prevents lipid accumulation in adipocytes by acting as PPARγ modulator (Granlund *et al*., 2003).

The joint up-regulation of PPARγ and FAS, ACC and LPL gene expression in bovine adipose tissue by propionate infusion (Lee and Hossner, 2002) is in favour of the implication of PPARγ in the nutritional or insulin activation of lipogenesis in ruminants, in adipose tissue at least. Elsewhere, bovine PPARγ1 and PPARγ2 cDNAs have been isolated and characterised (Sundvold *et al*., 1997). The distribution of PPARγ1 and PPARγ2 transcripts reveals a high expression in adipose tissue with equal quantities of both transcripts, and the PPARγ2 mRNA was also found at relatively high levels in spleen and lung and to a lesser extent in the mammary gland, ovary and small intestine. Moreover, Loor *et al.* (2003) analysed mammary gene expression in peripartal dairy cows using

a bovine cDNA microarray and showed an up-regulation of PPARγ gene expression between –14 and +14 days relative to parturition. Nevertheless, the role of PPARγ, as well as of PPARα or PPARβ, in the regulation of ruminant lipid metabolism has still to be defined.

Other transcription factors

The molecular mechanisms which control milk protein and lipogenic gene expression are not fully understood and probably involve undiscovered proteins within the mammary gland. Thus in the bovine mammary gland, Wheeler *et al.* (1997) identified the transcription factors Sp1 and NF-1, known in rodent to act in conjunction with other proteins such as SREBP1, and six nuclear proteins whose abundance was positively related with lactation or pregnancy status; four of these proteins were identified as lactoferrin, annexin II, vimentin and heavy-chain immunoglobulin. The presence of lactoferrin in the nuclear extracts was consistent with a recent proposal that lactoferrin binds to DNA in a sequence-specific manner and activates transcription (He and Furmanski, 1995). However the function of lactoferrin as a transcription factor has not yet been confirmed.

Elsewhere, over the past few years, response elements to lactogenic hormones have been mapped within the promoters of milk protein genes, and, in some cases, the proteins that mediate the lactational signals are known. Signal transducer(s) and activator(s) of transcription (Stat) form a family of seven distinct proteins. Structurally related transcription factors (Stat1-6, 5a and 5b) have been characterised. Stat5 was originally identified as a "mammary gland factor" mediating the prolactin signal to establish galactopoiesis (Rosen *et al*., 1999). In bovine, Stat5a and Stat5b genes have already been sequenced (Seyfert *et al*., 2000). Recently, Mao *et al.* (2002) characterised the bovine promoter III (PIII) of ACCα and demonstrated the presence of a transcription Stat-binding site at position –797, whose inactivation abolished lactogenic induction. Hence, this work provides the first evidence for the assumption that prolactin acting through Stat5 contributes to the activation of ACC expression which could contribute to the stimulation of milk fat synthesis during lactogenesis. Elsewhere, in bovine mammary gland explant culture, prolactin, growth hormone and IGF1 stimulated Stat5 DNA binding activity (Yang *et al*., 2000a). Furthermore, the same authors demonstrated that both Stat5 protein and Stat5 activity are modulated *in vivo* by several physiological signals, such as GH infusion and milking frequency (Yang *et al*., 2000b). Taken together, these data suggest (i) that Stat5 might be important in regulating mammary secretion by coordinating milk FA and protein synthesis during lactogenesis and/or galactopoiesis and (ii) that Stat5 transcription factor may represent part of the common route by which different extracellular signals linked to hormonal status as well as to milking frequency could converge and be transduced intracellularly to coordinate cell functions in the mammary gland. It is however not known if Stat proteins are involved in the nutritional regulation of mammary metabolism.

Conclusions and perspectives

Over the last decades, the biochemical pathways of lipid synthesis in the mammary gland have been elucidated, and the structures of many of the enzymatic proteins and their cDNAs have been resolved. This knowledge allowed the first studies on the nutritional regulation of a few "candidate" genes involved in mammary FA uptake (LPL), *de novo* synthesis (ACC and FAS) and desaturation (SCD). From these studies it was shown that the responses of mammary

"candidate" gene expression to nutritional factors are not always in accordance with milk FA secretion responses. Thus, in goat studies (Bernard *et al*., 2005b; Bernard *et al*., unpublished), mammary LPL mRNA and activity responses to diet were not related, and the strong increase in long-chain FA yield was generally not related with LPL activity. Both in goats and cows, *in vivo* studies suggest that the availability of substrates rather than the LPL activity is the limiting factor in the uptake of long-chain FA, except when extreme MFD diets are delivered to cows, in which both mammary LPL mRNA and activity decreased. Studies in cows and goats have shown that ACC gene expression is a key factor of short- and medium-chain FA synthesis, but is not always repressed by the addition of PUFAs to the diet, in goat at least. From goat studies, it was shown that ACC and FAS gene expression are regulated by dietary factors at a transcriptional level at least, and that SCD is regulated at a transcriptional and/or post-transcriptional level, depending on the lipid supplements. In cows, SCD mRNA abundance varied little among the diets studied, except for a decrease when "protected" fish oil was fed.

Because of the importance of the SCD enzyme in maintaining the fluidity of cellular membranes and milk fat (Chilliard *et al*., 2000; Parodi, 1982), it is likely that a fine balance between the exogenous unsaturated FA and the mammary SCD desaturation products must be maintained within the mammary gland.

Considering that only few data are available on the nutritional regulation of mammary gene expression, there is still a lot to be learnt about the regulatory systems, especially the intracellular signalling systems involved in these regulations. The basis of the effects of nutrients and particularly the identification of specific *trans*-FA controlling lipogenic gene expression are obvious targets. Thus, a high dose of *trans*-10, *cis*-12 CLA infused post-ruminally, acts as a potent inhibitor of the expression of all the lipogenic genes, but the level of this FA remains low in all feeding trials, including those with MFD diets, and sometimes does not increase at all. The effect of *trans*-10 C18:1, which largely responds to dietary factors, needs further investigation as its concentration is negatively related to milk fat response in cows and goats. However, marked differences are observed between the milk fat yield responses of these 2 species, despite large apparent similarities in milk FA profile responses. To better understand the molecular mechanisms involved in dietary and/or species related responses, more information on the promoter of the lipogenic genes should be acquired, which will help to clarify the roles and mechanisms of action of PUFA and/or *trans*-FA. Few data on the transcription factors are available, but have singled out the role of SREBP-1 for FA effects at least, and Stat5 for hormonal and physiological effects at least, whereas the roles of PPARs need to be unravelled. The answers to these questions are being delayed by the almost complete lack of *in vitro* systems that undergo the orchestrated events that allow synthesis and secretion of milk fat, and more efficient systems urgently need to be developed.

This review focused on the nutritional regulation of mammary lipogenic gene expression controlling lipid synthesis, and published studies were conducted mainly on 4 candidate genes only. However, the expression of specific proteins present on the milk fat globule membrane (Mather, 2000) such as butyrophilin, xanthine oxidoreductase and CD36, which intervene in milk lipid secretion, are also likely to have consequences on milk fat yield and composition (McManaman *et al*., 2002; Ogg *et al*., 2004). The current development of tools for studying the mammary transcriptome (macro- and micro-arrays; e.g. Leroux *et al*., 2003; Loor *et al*., 2003;

Suchyta *et al*., 2003) will allow study of the nutritional regulation of expression of a large number of new genes putatively involved in the lactating mammary gland function including milk lipid synthesis and secretion, and its FA profile. In addition, these tools could allow the regulation pathway and its actors to be deciphered. Moreover, further investigations on ruminal digestion and body metabolism of nutrients (absorption, partitioning between tissues) and mammary metabolic flows will also contribute to highlight the mechanisms underlying the observed responses to dietary factors, and the differences among ruminant species.

Acknowledgements

The authors thank P. Béraud for secretarial assistance during the preparation of the manuscript.

References

Abu-Elheiga, L., D.B. Almarza-Ortega, A. Baldini & S.J. Wakil (1997). Human acetyl-CoA carboxylase 2. Molecular cloning, characterization, chromosomal mapping, and evidence for two isoforms. Journal of Biological Chemistry, 272, 10669-77.

Abu-Elheiga, L., W.R. Brinkley, L. Zhong, S.S. Chirala, G. Woldegiorgis & S.J. Wakil (2000). The subcellular localization of acetyl-CoA carboxylase 2. Proceedings of the National Academy of Sciences USA, 97, 1444-9.

Abumrad, N.A., M.R. el-Maghrabi, E.Z. Amri, E. Lopez & P.A. Grimaldi (1993). Cloning of a rat adipocyte membrane protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. Homology with human CD36. Journal of Biological Chemistry, 268, 17665-8.

Ahnadi, C.E., N. Beswick, L. Delbecchi, J.J. Kennelly & P. Lacasse (2002). Addition of fish oil to diets for dairy cows. II. Effects on milk fat and gene expression of mammary lipogenic enzymes. Journal of Dairy Research, 69, 521-31.

Annison, E.F., J.L. Linzell & C.E. West (1968). Mammary and whole animal metabolism of glucose and fatty acids in fasting lactating goats. Journal of Physiology, 197, 445-59.

Aoki, N., T. Ishii, S. Ohira, Y. Yamaguchi, M. Negi, T. Adachi, R. Nakamura & T. Matsuda (1997). Stage specific expression of milk fat globule membrane glycoproteins in mouse mammary gland: comparison of MFG-E8, butyrophilin, and CD36 with a major milk protein, beta-casein. Biochimica et Biophysica Acta, 1334, 182-90.

Assumpcao, R.P., F.D. Santos, C.L. Setta, G.F. Barreto, I.E. Matta, D. Estadella, V.B. Azeredo & M.G. Tavares do Carmo (2002). Trans fatty acids in maternal diet may impair lipid biosynthesis in mammary gland of lactating rats. Annals of Nutrition and Metabolism, 46, 169-75.

Barber, M.C., R.A. Clegg, M.T. Travers & R.G. Vernon (1997). Lipid metabolism in the lactating mammary gland. Biochimica et Biophysica Acta, 1347, 101-26.

Barber, M.C.& M.T. Travers (1995). Cloning and characterisation of multiple acetyl-CoA carboxylase transcripts in ovine adipose tissue. Gene, 154, 271-5.

Barber, M.C.& M.T. Travers (1998). Elucidation of a promoter activity that directs the expression of acetyl-CoA carboxylase alpha with an alternative N-terminus in a tissue-restricted fashion. Biochemical Journal, 333, 17-25.

Barber, M.C., A.J. Vallance, H.T. Kennedy & M.T. Travers (2003). Induction of transcripts derived from promoter III of the acetyl-CoA carboxylase-alpha gene in mammary gland is associated with recruitment of SREBP-1 to a region of the proximal promoter defined by a DNase I hypersensitive site. Biochemical Journal, 375, 489-501.

Bauman D.E., B.A. Corl, L.H. Baumgard & J.M. Griinari (2001). Conjugated linoleic acid (CLA) and the dairy cow. In: J.W. P.C. Garnsworthy (ed.) Recent advances in animal nutrition, Nottingham, UK: Nottingham university press, 221-250.

Bauman, D.E.& J.M. Griinari (2001). Regulation and Nutritional Manipulation of Milk Fat: Low-Fat Milk Syndrome. Livestock Production Science, 70, 15-29.

Bauman, D.E.& J.M. Griinari (2003). Nutritional regulation of milk fat synthesis. Annual Review of Nutrition, 23, 203-27.

Baumgard, L.H., B.A. Corl, D.A. Dwyer, A. Saebo & D.E. Bauman (2000). Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis. American Journal of Physiology: Regulatory, Integrative and Comparative Physiology, 278, R179-84.

Baumgard, L.H., E. Matitashvili, B.A. Corl, D.A. Dwyer & D.E. Bauman (2002). trans-10, cis-12 conjugated linoleic acid decreases lipogenic rates and expression of genes involved in milk lipid synthesis in dairy cows. Journal of Dairy Science, 85, 2155-63.

Bernard, L., C. Leroux, M. Bonnet, J. Rouel, P. Martin & Y. Chilliard (2005a). Expression and nutritional regulation of lipogenic genes in mammary gland and adipose tissues of lactating goats. Journal of Dairy Research , 72, 250-5.

Bernard, L., C. Leroux, H. Hayes, M. Gautier, Y. Chilliard & P. Martin (2001). Characterization of the caprine stearoyl-CoA desaturase gene and its mRNA showing an unusually long 3'-UTR sequence arising from a single exon. Gene, 281, 53-61.

Bernard, L., J. Rouel, C. Leroux, A. Ferlay, Y. Faulconnier, P. Legrand & Y. Chilliard (2005b). Mammary lipid metabolism and fatty acid secretion in Alpine goats fed vegetable lipids. Journal of Dairy Science, 88, 1478-89.

Beswick, N.S.& J.J. Kennelly (1998). The influence of bovine growth hormone and growth hormone releasing factor on acetyl-CoA carboxylase and fatty acid synthase in primiparous Holstein cows. Comparative Biochemistry and Physiology. Part C. Pharmacology, Toxicology and Endocrinology, 120, 241-9.

Bickerstaffe, R.& E.F. Annison (1970). The desaturase activity of goat and sow mammary tissue. Comparative Biochemistry and Physiology, 35, 653-65.

Bonnet, M., Y. Faulconnier, J. Flechet, J.F. Hocquette, C. Leroux, D. Langin, P. Martin & Y. Chilliard (1998). Messenger RNAs encoding lipoprotein lipase, fatty acid synthase and hormone-sensitive lipase in the adipose tissue of underfed-refed ewes and cows. Reproduction Nutrition Development, 38, 297-307.

Bonnet, M., C. Leroux, Y. Chilliard & P. Martin (2000a). Rapid communication: nucleotide sequence of the ovine lipoprotein lipase cDNA. Journal of Animal Science, 78, 2994-5.

Bonnet, M., C. Leroux, Y. Faulconnier, J.F. Hocquette, F. Bocquier, P. Martin & Y. Chilliard (2000b). Lipoprotein lipase activity and mRNA are up-regulated by refeeding in adipose tissue and cardiac muscle of sheep. Journal of Nutrition, 130, 749-56.

Brown, M.S.& J.L. Goldstein (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell, 89, 331-40.

Camps, L., M. Reina, M. Llobera, S. Vilaro & T. Olivecrona (1990). Lipoprotein lipase: cellular origin and functional distribution. American Journal of Physiology, 258, C673-81.

Chilliard, Y.& A. Ferlay (2004). Dietary lipids and forages interactions on cow and goat milk fatty acid composition and sensory properties. Reproduction Nutrition Development, 44, 467-92.

Chilliard, Y., A. Ferlay & M. Doreau (2001). Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. Livestock Production Science, 70, 31-48.

Chilliard, Y., A. Ferlay, R.M. Mansbridge & M. Doreau (2000). Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, trans and conjugated fatty acids. Annales de Zootechnie, 49, 181-205.

Chilliard, Y., A. Ferlay, J. Rouel & G. Lamberet (2003a). A review of nutritional and physiological factors affecting goat milk lipid synthesis and lipolysis. Journal of Dairy Science, 86, 1751-70.

Chilliard, Y., G. Gagliostro, J. Flechet, J. Lefaivre & I. Sebastian (1991). Duodenal rapeseed oil infusion in early and midlactation cows. 5. Milk fatty acids and adipose tissue lipogenic activities. Journal of Dairy Science, 74, 1844-54.

Chilliard Y., J. Rouel, P. Capitan, J.M. Chabosseau, K. Raynal-Ljutovac & A. Ferlay (2003b). Correlations between milk fat content and fatty acid composition in goats receiving different combinations of forages and lipid supplements. In: van der Honing Y. (ed.) Book of abstracts of the 54th Annual Meeting of European Association for Animal Production, Rome, Italy, 31 August-3 september 2003, Wageningen Acad Publ (NL), p. 343.

Chilliard Y., J. Rouel, J.M. Chabosseau, P. Capitan, P. Gaborit & A. Ferlay (2003c). Interactions between raygrass preservation and linseed oil supplementation on goat milk yield and composition, including trans and conjugated fatty acids. In: van der Honing Y. (ed.) Book of abstracts of the 54th Annual Meeting of European Association for Animal Production, Rome, Italy, 31 August-3 september 2003, Wageningen Acad Publ (NL), p. 343.

Christie, W.W. (1979). The effects of diet and other factors on the lipid composition of ruminant tissues and milk. Progress in Lipid Research, 17, 245-77.

Chung, M., S. Ha, S. Jeong, J. Bok, K. Cho, M. Baik & Y. Choi (2000). Cloning and characterization of bovine stearoyl CoA desaturase l cDNA from adipose tissues. Bioscience Biotechnology and Biochemistry, 64, 1526-30.

Clarke, S.D. (2001). Nonalcoholic steatosis and steatohepatitis. I. Molecular mechanism for polyunsaturated fatty acid regulation of gene transcription. American Journal of Physiology: Gastrointestinal and Liver Physiology, 281, G865-9.

Clarke, S.D.& S. Abraham (1992). Gene expression: nutrient control of pre- and posttranscriptional events. FASEB Journal, 6, 3146-52.

Coleman, R.A.& D.P. Lee (2004). Enzymes of triacylglycerol synthesis and their regulation. Progress in Lipid Research, 43, 134-76.

Corl, B.A., L.H. Baumgard, D.A. Dwyer, J.M. Griinari, B.S. Phillips & D.E. Bauman (2001). The role of Delta(9)-desaturase in the production of cis-9, trans-11 CLA. Journal of Nutritional Biochemistry, 12, 622-30.

Corl, B.A., L.H. Baumgard, J.M. Griinari, P. Delmonte, K.M. Morehouse, M.P. Yurawecz & D.E. Bauman (2002). Trans-7,cis-9 CLA is synthesized endogenously by delta9-desaturase in dairy cows. Lipids, 37, 681-8.

Coulon, J.B., D. Dupont, S. Pochet, P. Pradel & H. Duployer (2001). Effect of genetic potential and level of feeding on milk protein composition. Journal of Dairy Research, 68, 569-577.

Davis C.L.& R.E. Brown (1970). Low-fat milk syndrome.Physiology of digestion and metabolism in ruminants, Newcastle upon Tyne, UK: Oriel, 545-565.

Del Prado, M., S. Villalpando, J. Gordillo & H. Hernandez-Montes (1999). A high dietary lipid intake during pregnancy and lactation enhances mammary gland lipid uptake and lipoprotein lipase activity in rats. Journal of Nutrition, 129, 1574-8.

Delbecchi, L., C.E. Ahnadi, J.J. Kennelly & P. Lacasse (2001). Milk fatty acid composition and mammary lipid metabolism in Holstein cows fed protected or unprotected canola seeds. Journal of Dairy Science, 84, 1375-81.

Ding, S.T., A.P. Schinckel, T.E. Weber & H.J. Mersmann (2000). Expression of porcine transcription factors and genes related to fatty acid metabolism in different tissues and genetic populations. Journal of Animal Science, 78, 2127-34.

Enoch, H.G., A. Catala & P. Strittmatter (1976). Mechanism of rat liver microsomal stearyl-CoA desaturase. Studies of the substrate specificity, enzyme-substrate interactions, and the function of lipid. Journal of Biological Chemistry, 251, 5095-103.

Ferlay A., J. Rouel, J.M. Chabosseau, P. Capitan, K. Raynal-Ljutovac & Y. Chilliard (2003). Interactions between raygrass preservation and high-oleic sunflower oil supplementation on goat milk composition, including trans and conjugated fatty acids. In: van der Honing Y. (ed.) Book of abstracts of the 54th Annual Meeting of European Association for Animal Production, Rome, Italy, 31 August-3 september 2003, Wageningen Acad Publ (NL), p. 350.

Gagliostro, G., Y. Chilliard & M.J. Davicco (1991). Duodenal rapeseed oil infusion in early and midlactation cows. 3. Plasma hormones and mammary apparent uptake of metabolites. Journal of Dairy Science, 74, 1893-903.

German J.B., C. Morand, C.J. Dillard & R. Xu (1997). Milk fat composition: Targets for alteration of function and nutrition. In: R.A.S. Welch, D.J.W. Burns, S.R. Davis, A.I. Popay, and C.G. Prosser (ed.) Milk composition, production and biotechnology. CAB international, New York, (NY), 39-72.

Girard, J., P. Ferre & F. Foufelle (1997). Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. Annual Review of Nutrition, 17, 325-52.

Girard, J., D. Perdereau, F. Foufelle, C. Prip-Buus & P. Ferre (1994). Regulation of lipogenic enzyme gene expression by nutrients and hormones. FASEB Journal, 8, 36-42.

Granlund, L., L.K. Juvet, J.I. Pedersen & H.I. Nebb (2003). Trans10, cis12-conjugated linoleic acid prevents triacylglycerol accumulation in adipocytes by acting as a PPARgamma modulator. Journal of Lipid Research, 44, 1441-52.

Griinari, J.M., B.A. Corl, S.H. Lacy, P.Y. Chouinard, K.V. Nurmela & D.E. Bauman (2000). Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Delta(9)-desaturase. Journal of Nutrition, 130, 2285-91.

Griinari, J.M., D.A. Dwyer, M.A. McGuire, D.E. Bauman, D.L. Palmquist & K.V. Nurmela (1998). Trans-octadecenoic acids and milk fat depression in lactating dairy cows. Journal of Dairy Science, 81, 1251-61.

Grisart, B., W. Coppieters, F. Farnir, L. Karim, C. Ford, P. Berzi, N. Cambisano, M. Mni, S. Reid, P. Simon, R. Spelman, M. Georges & R. Snell (2002). Positional candidate cloning of a QTL in dairy cattle: identification of a missense mutation in the bovine DGAT1 gene with major effect on milk yield and composition. Genome Research, 12, 222-31.

Grisart, B., F. Farnir, L. Karim, N. Cambisano, J.J. Kim, A. Kvasz, M. Mni, P. Simon, J.M. Frere, W. Coppieters & M. Georges (2004). Genetic and functional confirmation of the causality of the DGAT1 K232A quantitative trait nucleotide in affecting milk yield and composition. Proceedings of the National Academy of Sciences USA, 101, 2398-403.

Guichard, C., I. Dugail, X. Le Liepvre & M. Lavau (1992). Genetic regulation of fatty acid synthetase expression in adipose tissue: overtranscription of the gene in genetically obese rats. Journal of Lipid Research, 33, 679-87.

Hansen, H.O.& J. Knudsen (1987). Effect of exogenous long-chain fatty acids on lipid biosynthesis in dispersed ruminant mammary gland epithelial cells: esterification of long-chain exogenous fatty acids. Journal of Dairy Science, 70, 1344-9.

Hansen, H.O., D. Tornehave & J. Knudsen (1986). Synthesis of milk specific fatty acids and proteins by dispersed goat mammary-gland epithelial cells. Biochemical Journal, 238, 167-72.

He, J.& P. Furmanski (1995). Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. Nature, 373, 721-4.

Jayakumar, A., M.H. Tai, W.Y. Huang, W. al-Feel, M. Hsu, L. Abu-Elheiga, S.S. Chirala & S.J. Wakil (1995). Human fatty acid synthase: properties and molecular cloning. Proceedings of the National Academy of Sciences USA, 92, 8695-9.

Jayan, G.C.& J.H. Herbein (2000). "Healthier" dairy fat using trans-vaccenic acid. Nutrition and Food Science, 30, 304-309.

Jensen, D.R., D.H. Bessesen, J. Etienne, R.H. Eckel & M.C. Neville (1991). Distribution and source of lipoprotein lipase in mouse mammary gland. Journal of Lipid Research, 32, 733-42.

Jensen, R.G. (2002). The composition of bovine milk lipids: January 1995 to December 2000. Journal of Dairy Science, 85, 295-350.

Kaestner, K.H., J.M. Ntambi, T.J. Kelly Jr & M.D. Lane (1989). Differentiation-induced gene expression in 3T3-L1 preadipocytes. A second differentially expressed gene encoding stearoyl-CoA desaturase. Journal of Biological Chemistry, 264, 14755-61.

Kim, J.B., G.D. Spotts, Y.D. Halvorsen, H.M. Shih, T. Ellenberger, H.C. Towle & B.M. Spiegelman (1995). Dual DNA binding specificity of ADD1/SREBP1 controlled by a single amino acid in the basic helix-loop-helix domain. Molecular and Cellular Biology, 15, 2582-8.

Kinsella, J.E. (1970). Stearic acid metabolism by mammary cells. Journal of Dairy Science, 53, 1757-65.

Kirchgessner, T.G., K.L. Svenson, A.J. Lusis & M.C. Schotz (1987). The sequence of cDNA encoding lipoprotein lipase. A member of a lipase gene family. Journal of Biological Chemistry, 262, 8463-6.

Knudsen, J.& I. Grunnet (1982). Transacylation as a chain-termination mechanism in fatty acid synthesis by mammalian fatty acid synthetase. Synthesis of medium-chain-length (C8-C12) acyl-CoA esters by goat mammary gland fatty acid synthetase. Biochemical Journal, 202, 139-43.

Knudsen, J., T.B. Neergaard, B. Gaigg, M.V. Jensen & J.K. Hansen (2000). Role of acyl-CoA binding protein in acyl-CoA metabolism and acyl-CoA-mediated cell signaling. Journal of Nutrition, 130, 294S-8S.

Lee, S.H.& K.L. Hossner (2002). Coordinate regulation of ovine adipose tissue gene expression by propionate. Journal of Animal Science, 80, 2840-9.

Legrand, P., D. Catheline, M.C. Fichot & P. Lemarchal (1997). Inhibiting delta9-desaturase activity impairs triacylglycerol secretion in cultured chicken hepatocytes. Journal of Nutrition, 127, 249-56.

Lehner, R.& A. Kuksis (1996). Biosynthesis of triacylglycerols. Progress in Lipid Research, 35, 169-201.

Leroux, C., J. Laubier, C. Giraud-Delville, F. Le Provost, S. Chadi, L. Bernard, M. Bonnet, Y. Chilliard & P. Martin (2005). Sharp increase of goat fatty acid synthase gene expression in mammary gland during the last part of pregnancy, Journal of Dairy Research, submitted.

Leroux, C., F. Le Provost, E. Petit, L. Bernard, Y. Chilliard & P. Martin (2003). Real-time RT-PCR and cDNA macroarray to study the impact of the genetic polymorphism at the alphas1-casein locus on the expression of genes in the goat mammary gland during lactation. Reproduction Nutrition Development, 43, 459-69.

Lin, X., J.J. Loor & J.H. Herbein (2004). Trans10,cis12-18:2 is a more potent inhibitor of de novo fatty acid synthesis and desaturation than cis9,trans11-18:2 in the mammary gland of lactating mice. Journal of Nutrition, 134, 1362-8.

Loor, J.J., H.M. Dann, R.E. Everts, S.L. Rodriguez-Zas, H.A. Lewin & J.K. Drackley (2003). Mammary and hepatic gene expression analysis in peripartal dairy cows using a bovine cDNA microarray. Journal of Animal Science, 82, 196.

Loor, J.J., A. Ferlay, A. Ollier, M. Doreau & Y. Chilliard (2005a). Relationship among trans and conjugated fatty acids and bovine milk fat yield due to dietary concentrate and linseed oil. Journal of Dairy Science, 88, 726-40.

Loor, J.J., M. Doreau, J.M. Chardigny, A. Ollier, J.L. Sebedio & Y. Chilliard (2005b). Effects of ruminal or duodenal supply of fish oil on milk fat secretion and profiles of trans-fatty acids and conjugated linoleic acid isomers in dairy cows fed maize silage. Animal Feed Science and Technology, 119, 227-46.

Loor, J.J.& J.H. Herbein (2003). Reduced fatty acid synthesis and desaturation due to exogenous trans10,cis12-CLA in cows fed oleic or linoleic oil. Journal of Dairy Science, 86, 1354-69.

Loor, J.J., K. Ueda, A. Ferlay, Y. Chilliard & M. Doreau (2004a). Biohydrogenation, duodenal flow, and intestinal digestibility of trans fatty acids and conjugated linoleic acids in response to dietary forage: concentrate ratio and linseed oil in dairy cows. Journal of Dairy Science, 87, 2472-85.

Loor, J.J., K. Ueda, A. Ferlay, Y. Chilliard & M. Doreau (2004b). Short communication: Diurnal profiles of conjugated linoleic acids and trans fatty acids in ruminal fluid from cows fed a high concentrate diet supplemented with fish oil, linseed oil, or sunflower oil. Journal of Dairy Science, 87, 2468-71.

Lopez-Casillas, F., M.V. Ponce-Castaneda & K.H. Kim (1991). In vivo regulation of the activity of the two promoters of the rat acetyl coenzyme-A carboxylase gene. Endocrinology, 129, 1049-58.

Makowski, L.& G.S. Hotamisligil (2004). Fatty acid binding proteins-the evolutionary crossroads of inflammatory and metabolic responses. Journal of Nutrition, 134, 2464S-2468S.

Mao, J., S. Marcos, S.K. Davis, J. Burzlaff & H.M. Seyfert (2001). Genomic distribution of three promoters of the bovine gene encoding acetyl-CoA carboxylase alpha and evidence that the nutritionally regulated promoter I contains a repressive element different from that in rat. Biochemical Journal, 358, 127-35.

Mao, J., A.J. Molenaar, T.T. Wheeler & H.M. Seyfert (2002). STAT5 binding contributes to lactational stimulation of promoter III expressing the bovine acetyl-CoA carboxylase alpha-encoding gene in the mammary gland. Journal of Molecular Endocrinology, 29, 73-88.

Marshall, M.O.& J. Knudsen (1977). The specificity of 1-acyl-sn-glycerol 3-phosphate acyltransferase in microsomal fractions from lactating cow mammary gland towards short, medium and long chain acyl-CoA esters. Biochimica et Biophysica Acta, 489, 236-41.

Massaro, M., M.A. Carluccio & R. De Caterina (1999). Direct vascular antiatherogenic effects of oleic acid: a clue to the cardioprotective effects of the Mediterranean diet. Cardiologia, 44, 507-513.

Mather, I.H. (2000). A review and proposed nomenclature for major proteins of the milk-fat globule membrane. Journal of Dairy Science, 83, 203-47.

Matitashvili, E.& D.E. Bauman (2000). Effect of different isomers of C18:1 and C18:2 fatty acids on lipogenesis in bovine mammary epithelial cells. Journal of Animal Science, 78, 165.

Mayorek, N., I. Grinstein & J. Bar-Tana (1989). Triacylglycerol synthesis in cultured rat hepatocytes. The rate-limiting role of diacylglycerol acyltransferase. European Journal of Biochemistry, 182, 395-400.

McManaman, J.L., C.A. Palmer, R.M. Wright & M.C. Neville (2002). Functional regulation of xanthine oxidoreductase expression and localization in the mouse mammary gland: evidence of a role in lipid secretion. Journal of Physiology, 545, 567-79.

Mihara, K. (1990). Structure and regulation of rat liver microsomal stearoyl-CoA desaturase gene. Journal of Biochemistry (Tokyo), 108, 1022-9.

Mikkelsen, J.& J. Knudsen (1987). Acyl-CoA-binding protein from cow. Binding characteristics and cellular and tissue distribution. Biochemical Journal, 248, 709-14.

Mistry, D.H.& J.F. Medrano (2002). Cloning and localization of the bovine and ovine lysophosphatidic acid acyltransferase (LPAAT) genes that codes for an enzyme involved in triglyceride biosynthesis. Journal of Dairy Science, 85, 28-35.

Molenaar, A., J. Mao, K. Oden & H.M. Seyfert (2003). All three promoters of the acetyl-coenzyme A-carboxylase alpha-encoding gene are expressed in mammary epithelial cells of ruminants. Journal of Histochemistry and Cytochemistry, 51, 1073-81.

Mosley, E.E., G.L. Powell, M.B. Riley & T.C. Jenkins (2002). Microbial biohydrogenation of oleic acid to trans isomers in vitro. Journal of Lipid Research, 43, 290-6.

Neville, M.C.& M.F. Picciano (1997). Regulation of milk lipid secretion and composition. Annual Review of Nutrition, 17, 159-83.

Nielsen, M.O.& K. Jakobsen (1994). Changes in mammary uptake of free fatty acids, triglyceride, cholesterol and phospholipid in relation to milk synthesis during lactation in goats. Comparative Biochemistry and Physiology. A. Comparative Physiology, 109, 857-67.

Ntambi, J.M. (1999). Regulation of stearoyl-CoA desaturase by polyunsaturated fatty acids and cholesterol. Journal of Lipid Research, 40, 1549-58.

Ntambi, J.M., S.A. Buhrow, K.H. Kaestner, R.J. Christy, E. Sibley, T.J. Kelly Jr & M.D. Lane (1988). Differentiation-induced gene expression in 3T3-L1 preadipocytes. Characterization of a differentially expressed gene encoding stearoyl- CoA desaturase. Journal of Biological Chemistry, 263, 17291-300.

Offer, N.W., B.K. Speake, J. Dixon & M. Marsden (2001). Effect of fish-oil supplementation on levels of (n-3) poly-unsaturated fatty acids in the lipoprotein fractions of bovine plasma. Animal Science, 73, 523-531.

Ogg, S.L., A.K. Weldon, L. Dobbie, A.J. Smith & I.H. Mather (2004). Expression of butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets. Proceedings of the National Academy of Sciences USA, 101, 10084-9.

Palmquist, D.L., A.D. Beaulieu & D.M. Barbano (1993). Feed and animal factors influencing milk fat composition. Journal of Dairy Science, 76, 1753-71.

Pariza, M.W., Y. Park & M.E. Cook (2001). The biologically active isomers of conjugated linoleic acid. Progress in Lipid Research, 40, 283-98.

Parodi, P.W. (1982). Positional distribution of fatty acids in triglycerides from milk of several species of mammals. Lipids, 17, 437-42.

Perfield, J.W. 2nd, A. Saebo & D.E. Bauman (2004). Use of conjugated linoleic acid (CLA) enrichments to examine the effects of trans-8, cis-10 CLA, and cis-11, trans-13 CLA on milk-fat synthesis. Journal of Dairy Science, 87, 1196-202.

Peterson, D.G., E.A. Matitashvili & D.E. Bauman (2003). Diet-induced milk fat depression in dairy cows results in increased trans-10, cis-12 CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis. Journal of Nutrition, 133, 3098-102.

Peterson, D.G., E.A. Matitashvili & D.E. Bauman (2004). The inhibitory effect of trans-10, cis-12 CLA on lipid synthesis in bovine mammary epithelial cells involves reduced proteolytic activation of the transcription factor SREBP-1. Journal of Nutrition, 134, 2523-7.

Piperova, L.S., B.B. Teter, I. Bruckental, J. Sampugna, S.E. Mills, M.P. Yurawecz, J. Fritsche, K. Ku & R.A. Erdman (2000). Mammary lipogenic enzyme activity, trans fatty acids and conjugated linoleic acids are altered in lactating dairy cows fed a milk fat- depressing diet. Journal of Nutrition, 130, 2568-74.

Rosen, E.D., P. Sarraf, A.E. Troy, G. Bradwin, K. Moore, D.S. Milstone, B.M. Spiegelman & R.M. Mortensen (1999). PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Molecular Cell, 4, 611-7.

Rosen, E.D.& B.M. Spiegelman (2001). PPARgamma : a nuclear regulator of metabolism, differentiation, and cell growth. Journal of Biological Chemistry, 276, 37731-4.

Rouel J., A. Ferlay, E. Bruneteau, P. Capitan, K. Raynal-Ljutovac & Y. Chilliard (2004). Interactions between starchy concentrate and linseed oil supplementation on goat milk yield and composition, including trans and conjugated fatty acids (FA). In: van der Honing Y. (ed.) Book of abstracts of the 55th Annual Meeting of European Association for Animal Production, Bled, Slovenia, 5-9 september 2004, Wageningen Acad Publ (NL), p.124.

Roy, A., A. Ferlay, A. Ollier, P. Capitan, J.L. Sebedio & Y. Chilliard (2004). Cinétiques de réponse des acides gras trans et de l'acide ruménique du lait de vache à différents changements de régime alimentaires (Kinetics of response of milk trans fatty acids and rumenic acid to different dietary changes in cow). Nutrition Clinique et Métabolisme, 18, S34.

Sakai, J., E.A. Duncan, R.B. Rawson, X. Hua, M.S. Brown & J.L. Goldstein (1996). Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment. Cell, 85, 1037-46.

Sarraf, P., E. Mueller, W.M. Smith, H.M. Wright, J.B. Kum, L.A. Aaltonen, A. de la Chapelle, B.M. Spiegelman & C. Eng (1999). Loss-of-function mutations in PPAR gamma associated with human colon cancer. Molecular Cell, 3, 799-804.

Schoonjans, K., J. Peinado-Onsurbe, A.M. Lefebvre, R.A. Heyman, M. Briggs, S. Deeb, B. Staels & J. Auwerx (1996a). PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. EMBO Journal, 15, 5336-48.

Schoonjans, K., B. Staels & J. Auwerx (1996b). Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. Journal of Lipid Research, 37, 907-25.

Schweizer, M., K. Takabayashi, T. Laux, K.F. Beck & R. Schreglmann (1989). Rat mammary gland fatty acid synthase: localization of the constituent domains and two functional polyadenylation/termination signals in the cDNA. Nucleic Acids Research, 17, 567-86.

Senda, M., K. Oka, W.V. Brown, P.K. Qasba & Y. Furuichi (1987). Molecular cloning and sequence of a cDNA coding for bovine lipoprotein lipase. Proceedings of the National Academy of Sciences USA, 84, 4369-73.

Seyfert, H.M., C. Pitra, L. Meyer, R.M. Brunner, T.T. Wheeler, A. Molenaar, J.Y. McCracken, J. Herrmann, H.J. Thiesen & M. Schwerin (2000). Molecular characterization of STAT5A- and STAT5B-encoding genes reveals extended intragenic sequence homogeneity in cattle and mouse and different degrees of divergent evolution of various domains. Journal of Molecular Evolution, 50, 550-61.

Shimomura, I., H. Shimano, B.S. Korn, Y. Bashmakov & J.D. Horton (1998). Nuclear sterol regulatory element-binding proteins activate genes responsible for the entire program of unsaturated fatty acid biosynthesis in transgenic mouse liver. Journal of Biological Chemistry, 273, 35299-306.

Shimomura, I., H. Shimano, J.D. Horton, J.L. Goldstein & M.S. Brown (1997). Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. Journal of Clinical Investigation, 99, 838-45.

Shirley, J.E., R.S. Emery, E.M. Convey & W.D. Oxender (1973). Enzymic changes in bovine adipose and mammary tissue, serum and mammary tissue hormonal changes with initiation of lactation. Journal of Dairy Science, 56, 569-74.

Singh, K., D.G. Hartley, T.B. McFadden & D.D. Mackenzie (2004). Dietary fat regulates mammary stearoyl coA desaturase expression and activity in lactating mice. Journal of Dairy Research, 71, 1-6.

Small, D.M. (1991). The effects of glyceride structure on absorption and metabolism. Annual Review of Nutrition, 11, 413-34.

Specht, B., N. Bartetzko, C. Hohoff, H. Kuhl, R. Franke, T. Borchers & F. Spener (1996). Mammary derived growth inhibitor is not a distinct protein but a mix of heart-type and adipocyte-type fatty acid-binding protein. Journal of Biological Chemistry, 271, 19943-9.

Spitsberg, V.L., E. Matitashvili & R.C. Gorewit (1995). Association and coexpression of fatty-acid-binding protein and glycoprotein CD36 in the bovine mammary gland. European Journal of Biochemistry, 230, 872-8.

Suchyta, S.P., S. Sipkovsky, R.G. Halgren, R. Kruska, M. Elftman, M. Weber-Nielsen, M.J. Vandehaar, L. Xiao, R.J. Tempelman & P.M. Coussens (2003). Bovine mammary gene expression profiling using a cDNA microarray enhanced for mammary-specific transcripts. Physiological Genomics, 16, 8-18.

Sundvold, H., A. Brzozowska & S. Lien (1997). Characterisation of bovine peroxisome proliferator-activated receptors gamma 1 and gamma 2: genetic mapping and differential expression of the two isoforms. Biochemical and Biophysical Research Communications, 239, 857-61.

Wakil, S.J. (1989). Fatty acid synthase, a proficient multifunctional enzyme. Biochemistry, 28, 4523-30.

Wang, X., M.R. Briggs, X. Hua, C. Yokoyama, J.L. Goldstein & M.S. Brown (1993). Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. II. Purification and characterization. Journal of Biological Chemistry, 268, 14497-504.

Ward, R.J., M.T. Travers, S.E. Richards, R.G. Vernon, A.M. Salter, P.J. Buttery & M.C. Barber (1998). Stearoyl-CoA desaturase mRNA is transcribed from a single gene in the ovine genome. Biochimica et Biophysica Acta, 1391, 145-56.

West, C.E., R. Bickerstafee, E.F. Annison & J.L. Linzell (1972). Studies on the mode of uptake of blood triglycerides by the mammary gland of the lactating goat. The uptake and incorporation into milk fat and mammary lymph of labelled glycerol, fatty acids and triglycerides. Biochemical Journal, 126, 477-90.

Wheeler, T.T., M.K. Broadhurst, G.H. Rajan & R.J. Wilkins (1997). Differences in the abundance of nuclear proteins in the bovine mammary gland throughout the lactation and gestation cycles. Journal of Dairy Science, 80, 2011-9.

Williams, C.M. (2000). Dietary fatty acids and human health. Annales de Zootechnie, 49, 165-180.

Winter, A., W. Kramer, F.A. Werner, S. Kollers, S. Kata, G. Durstewitz, J. Buitkamp, J.E. Womack, G. Thaller & R. Fries (2002). Association of a lysine-232/alanine polymorphism in a bovine gene encoding acyl-CoA:diacylglycerol acyltransferase (DGAT1) with variation at a quantitative trait locus for milk fat content. Proceedings of the National Academy of Sciences USA, 99, 9300-5.

Yahyaoui, M.H., D. Vaiman, A. Sanchez & J.M. Folch (2003). Mapping of the goat stearoyl coenzyme A desaturase gene to chromosome 26. Animal Genetics, 34, 474-5.

Yang, J., J.J. Kennelly & V.E. Baracos (2000a). The activity of transcription factor Stat5 responds to prolactin, growth hormone, and IGF-I in rat and bovine mammary explant culture. Journal of Animal Science, 78, 3114-25.

Yang, J., J.J. Kennelly & V.E. Baracos (2000b). Physiological levels of Stat5 DNA binding activity and protein in bovine mammary gland. Journal of Animal Science, 78, 3126-34.

Yeldandi, A.V., M.S. Rao & J.K. Reddy (2000). Hydrogen peroxide generation in peroxisome proliferator-induced oncogenesis. Mutation Research, 448, 159-77.

Zhang, L., L. Ge, S. Parimoo, K. Stenn & S.M. Prouty (1999). Human stearoyl-CoA desaturase: alternative transcripts generated from a single gene by usage of tandem polyadenylation sites. Biochemical Journal, 340, 255-64.

Roles of growth hormone and leptin in the periparturient dairy cow

Y.R. Boisclair, S.R. Wesolowski, J.W. Kim and R.A. Ehrhardt
Cornell University, Department of Animal Science, 259 Morrison Hall, Ithaca, NY 14850, USA

Abstract

Dairy cattle experience dramatic changes in metabolism during the period from pregnancy to lactation when they enter a period of severe nutritional insufficiency. The energy shortfall of early lactation is met by mobilization of endogenous reserves, and by shifting the pattern of nutrients used by non-mammary tissues. These adaptations are coordinated by homeorhetic regulators such as growth hormone. Paradoxically, some of the actions of growth hormone, such as stimulation of plasma IGF-I, are attenuated in early lactation. This first part of this review will discuss recent molecular work aimed at identifying the basis for this phenomenon. Given the importance of metabolic homeostasis, other homeorhetic regulators are likely to be involved. A protein that was still unknown when the concept of homeorhesis was proposed is leptin, a hormone secreted by adipose tissue. Emerging evidence from other species suggests that leptin prioritizes the use of available energy among tissues during periods of nutritional insufficiency, consistent with homeorhetic actions. In the second part of this review, we will review data suggesting the possibility that leptin is a homeorhetic regulator in ruminants.

Keywords: homeorhesis, pregnancy, lactation, metabolism, ruminant

Introduction

In high yielding dairy cows, the onset of copious milk secretion increases the total energy requirements approximately four fold relative to late parturition (Bell, 1995; Drackley *et al*., 2001). These lactational demands occur in the absence of an adequate increase in feed intake. As a result, early lactating dairy cows suffer from substantial deficits in energy and specific nutrients limiting milk synthesis (Bell, 1995; Drackley *et al*., 2001). These shortfalls are met by mobilization of endogenous reserves, and by shifting the profile of nutrients consumed by non-mammary tissues (Bauman and Currie, 1980; Bell, 1995).

Mechanisms orchestrating these adaptations have been the subject of intense investigation over the last two decades (Bauman and Currie, 1980; Bell and Bauman, 1997; Vernon and Pond, 1997). These efforts have been motivated by two major considerations. First, dairy cows offer a unique model to study metabolic changes during the transition from an energy-sufficient to an energy-insufficient state (late pregnancy *vs.* early lactation). Second, failure in one or more metabolic adaptations is thought to underlie a major portion of the increased susceptibility of early lactating dairy cows to metabolic diseases and associated disorders (Goff and Horst, 1997; Ingvartsen *et al*., 2003).

In 1980, Bauman and Currie reviewed the role of hormones in orchestrating chronic changes in metabolism such as those seen during pregnancy and lactation, and coined the term homeorhesis to describe this type of regulation (Bauman and Currie, 1980). Among the hormones they

considered, only growth hormone (GH) has since been shown to be capable of homeorhetic actions in dairy cows [reviewed in (Bauman, 2000)]. An observation that has not been adequately explained, however, is the loss of some GH actions in early lactation. In the first part of this review, we will discuss recent work providing a mechanistic basis for this observation. We will then consider the possibility that the adipocyte-derived hormone leptin, discovered only in 1994, could act as a homeorhetic regulator of metabolism.

Growth hormone

Impaired GH-dependent milk yield and plasma IGF-I responses in early lactation

Current models ascribe a primary role to GH in coordinating metabolism during the transition from pregnancy to early lactation (Bauman, 2000; Bell, 1995). Consistent with this notion, the plasma concentration of GH is increased around parturition, and remains elevated in early lactation (Bell, 1995; Block *et al*., 2001). However, the positive galactopoietic effects of exogenous GH are weaker in early than post-peak lactating dairy cows (Bauman, 1999; Richard *et al*., 1985).

Organismal effects of GH are the sum of direct actions via its cognate receptor and indirect actions via increased IGF-I synthesis (Le Roith *et al*., 2001). The origin of the IGF-I mediating the indirect effects of GH is controversial. The traditional view is that the indirect effects are conveyed by plasma IGF-I produced in liver in a GH-dependent manner (Bauman and Vernon, 1993; Le Roith *et al*., 2001). This view has been questioned because liver IGF-I deficient mice appear to have normal growth and development despite low plasma IGF-I (Sjogren *et al*., 1999; Yakar *et al*., 1999). However, plasma IGF-I is necessary for complete body growth and bone development, even in liver IGF-I deficient mice (Ueki *et al*., 2000; Yakar *et al*., 2002). Therefore, liver-derived IGF-I is likely to mediate at least a portion of the effect of GH on peripheral tissues, including the mammary gland.

Significantly, early lactating dairy cows have depressed plasma IGF-I (Block *et al*., 2001; Kobayashi *et al*., 1999a), and lose the ability to mount a robust GH-dependent increase in plasma IGF-I (Ronge and Blum, 1989; Vicini *et al*., 1991). This is potentially an important defect as fully differentiated mammary epithelial cells have an abundant population of IGF-I receptor (Collier *et al*., 1993; Dehoff *et al*., 1988). These receptors are functionally important as shown by increased milk synthesis in ruminants after close arterial infusion of IGF-I (Prosser *et al*., 1994). IGF-I also activates signaling cascades in bovine mammary cells that lead to increased metabolic activities and cell number [e.g., proliferation and survival] (Hadsell *et al*., 2002; Shamay *et al*., 1988). The growth hormone receptor (GHR) has also been detected in mammary epithelial cells by *in situ* hybridization and immunohistochemistry (Plath-Gabler *et al*., 2001; Sinowatz *et al*., 2000). However, specific GH binding is negligible in the bovine mammary gland (Akers, 1985; Collier *et al*., 1993) and mammary epithelial cells have low expression of the IGF-I gene (Plath-Gabler *et al*., 2001). Overall, these data suggest that the impairment of GH-dependent synthesis of IGF-I by liver is partly responsible for poor milk yield responses after chronic GH treatment of early lactating dairy cows.

Basis for impaired IGF-I production in early lactation

Low plasma IGF-I in early lactation suggests impaired hepatic GH signaling. In general, receptor abundance is an important determinant of hormone responsiveness, and is regulated at the level of transcription. The bovine GHR gene contains 9 coding exons (exons 2 to 10) and is estimated to span at least 110 kb (Lucy *et al*., 2001). The upstream regulatory region of the bovine GHR gene contains at least 3 promoters, termed P1, P2 and P3 (Jiang and Lucy, 2001a; Jiang *et al*., 2000; Jiang *et al*., 1999). They initiate transcription from Exon 1A (promoter P1), Exon 1B (P2) and Exon1C (P3). The unique exons 1A, 1B and 1C are spliced onto a common core transcript composed of exons 2 to 10, giving rise to three major classes of transcripts designated as GHR1A, GHR1B and GHR1C transcripts (Jiang and Lucy, 2001b). Despite differences in their 5' region, all of these transcripts encode an identical GHR protein.

The GHR1A transcript is found only in postnatal liver and accounts for ~50% of total hepatic GHR mRNA in well-fed prepartum dairy cows (Kobayashi *et al*., 1999a; Lucy *et al*., 2001). Its abundance drops by > 50 % at parturition, but recovers substantially over the first 2 weeks of lactation (Kobayashi *et al*., 1999a; Radcliff *et al*., 2003b). GHR abundance, measured directly or inferred from GH binding, mimicked almost exactly the GHR1A transcript profile (Kim *et al*., 2004; Radcliff *et al*., 2003a). These data are consistent with a model whereby decreased GHR1A expression accounts for decreased GHR abundance, and therefore for reduced synthesis of IGF-I in periparturient dairy cows (Kim *et al*., 2004; Radcliff *et al*., 2003a; Radcliff *et al*., 2003b).

Factors responsible for decreased abundance of the GHR1A transcript in periparturient liver remained poorly understood. Metabolically, the periparturient period is dominated by the onset of severe undernutrition. This is reflected by reduced plasma concentrations of insulin and glucose, and increased concentration of NEFA (Block *et al*., 2001; Rhoads *et al*., 2004). Consistent with the involvement of insulin, chronic hyperinsulinemic-euglycemic clamps performed in either late pregnancy (day 26-29 prepartum) or early lactation (day 10-13 of lactation) increased indices of GHR abundance (GHR1A expression and GHR protein) and hepatic IGF-I production (IGF-I mRNA and plasma IGF-I) (Butler *et al*., 2003; Rhoads *et al*., 2004). The GHR promoter responsible for GHR1A synthesis (P1 promoter) is positively regulated by the binding of the liver-enriched transcription factor HNF4 to a proximal cis-element (Jiang and Lucy, 2001a). However, variation in the abundance of hepatic HNF4 does not explain the effects of parturition or insulin on GHR1A abundance in dairy cows (Kim *et al*., 2004; Rhoads *et al*., 2004). The same cis-element binds the transcription factor COUP-TFII (Xu *et al*., 2004), but whether parturition or insulin regulates the abundance of this transcription factor has not been reported. The periparturient period is also characterized by abrupt changes in the plasma concentrations of reproductive (progesterone and oestradiol) and stress hormones (cortisol and epinephrine) (Bell, 1995). When tested alone, none of these hormones has an effect on GHR1A abundance (Kobayashi *et al*., 1999b; Kobayashi *et al*., 2002). Alternatively, the signal triggering reduced hepatic GHR1A could be the interaction between two or more of the concentration changes observed for these hormones around parturition. However, this appears unlikely because periparturient beef cattle do not suffer from a reduction in GHR1A abundance (Jiang *et al*., 2005).

GH actions in adipose tissue and skeletal muscle during the periparturient period

Increased milk production requires metabolic actions of GH in adipose tissue and skeletal muscle (Bauman, 2000; Etherton and Bauman, 1998). These actions have been studied almost exclusively during the lactation interval following peak yield (Bauman, 2000; Etherton and Bauman, 1998). They include decreased peripheral glucose utilization (inferred to represent mostly decreased skeletal muscle uptake) and increased export of lipids from adipose tissue (Bauman, 2000; Bauman *et al*., 1988; Etherton and Bauman, 1998). These metabolic actions are best understood in adipose tissue where GH attenuates the lipogenic response to the hormone insulin and simultaneously amplifies the lipolytic response to β-adrenergic signals (Bauman, 2000; Sechen *et al*., 1990; Vernon, 2000). In general, these adaptations spare non-mammary utilization of limiting nutrients such as glucose and increase their availability for milk synthesis (Bauman, 2000; Bell, 1995).

Current evidence indicates that metabolic effects of GH are mediated directly in these tissues. First, mature adipocytes are devoid of IGF-I receptor, negating any GH effects via IGF-I (Caro *et al*., 1988; Vernon and Flint, 1989). Because skeletal muscles have receptors for both IGF-I and GH, metabolic actions could theoretically be mediated by either peptide (Le Roith *et al*., 2001; Singleton and Feldman, 2001). However, IGF-I increases rather than decreases glucose uptake in skeletal muscle (Fryburg *et al*., 1995; Singleton and Feldman, 2001). Recent work in engineered mouse models unambiguously demonstrates that GH attenuates insulin-mediated signaling and glucose utilization in skeletal muscle (Yakar *et al*., 2004)

Theoretically, poor galactopoietic responses in early lactation could also reflect impaired GH responses in skeletal muscle and in adipose tissue. There are important gaps in our understanding of GH receptor regulation and GH actions in these tissues, particularly in early lactation. GHR1B and GHR1C transcripts account for approximately 70% and 30% of all GHR transcripts in both tissues (Jiang and Lucy, 2001b; Jiang *et al*., 1999). These transcripts are not regulated during the transition period in either tissue when assessed collectively (Kim *et al*., 2004; Rhoads *et al*., 2004). Despite lack of mRNA regulation, GHR abundance in adipose tissue is higher in late pregnancy than in early lactation, and is increased by insulin in both states (Kim *et al*., 2004; Rhoads *et al*., 2004). These data raise the possibility that variation in GH responsiveness also occurs in non-hepatic tissues during the transition from pregnancy to lactation.

Leptin

Adipose tissue is the primary source of leptin

The discovery of leptin represents the culmination of work aimed at explaining the basis for the extreme appetite and obesity of the *ob/ob* mouse strain (Zhang *et al*., 1994). In these mice, the ob gene contains a mutation resulting in the absence of the corresponding protein. Treatment of *ob/ob* mice with recombinant ob protein normalizes appetite, body weight and a range of other defects [e.g., reproduction and immunity] (Chehab *et al*., 1996; Halaas *et al*., 1995; Lord *et al*., 1998). This protein was subsequently named leptin from "leptos", the Greek word for thin.

The leptin cDNA has been cloned for most farm animals, including cattle and sheep [reviewed in (Ingvartsen and Boisclair, 2001)]. Leptin is encoded by a single transcript of ~4.5 kb expressed primarily by adipose tissue. As shown for other species (Ahima and Flier, 2000), non-adipose sites of synthesis have also been detected in ruminants. For example, fetal sheep have significant leptin expression in the brain and liver (Ehrhardt *et al*., 2002). Similarly, in cattle, leptin is expressed in the rumen, abomasum and duodenum before weaning but only in the duodenum after weaning (Yonekura *et al*., 2002). The ruminant mammary epithelial cells also synthesizes leptin during pregnancy and during established lactation (Bonnet *et al*., 2002; Laud *et al*., 1999; Leury *et al*., 2003). Nevertheless, it is clear that the contribution of non-adipose sites to the pool of circulating leptin is negligible in adult ruminants.

In cattle and sheep, translation of the leptin mRNA produces a pre-protein of 167 amino acids and a mature protein of 146 amino acids after removal of the signal peptide (Dyer *et al*., 1997; Ji *et al*., 1998). Analysis of the human protein revealed a four-helix bundle structure characteristic of the long chain helical cytokine family, which includes GH (Zhang *et al*., 1997). The leptin protein from all known animals contains two cysteine residues (residues 96 and 146 in mature bovine leptin) forming a disulfide bridge required for bioactivity (Zhang *et al*., 1997).

Leptin acts predominantly on the central nervous system

Target tissues can be identified by considering the type and distribution of leptin receptors. In rodents, alternative splicing of the single leptin receptor gene produces at least 6 mRNAs (Ahima and Flier, 2000; Ingvartsen and Boisclair, 2001). They encode leptin receptor isoforms sharing an extracellular ligand binding domain of ~840 amino acid residues but having different intracellular domains. The longest isoform, Ob-Rb, has an intracellular domain of approximately 300 amino acids (Chen *et al*., 1996; Lee *et al*., 1996). Ob-Rb is capable of activating all known transduction signals attributed to leptin, including the JAK2-STAT3, MAPK and IRS-PI3 kinase pathways (Ahima and Flier, 2000; Myers, 2004). In rodents, Ob-Rb is expressed predominantly in hypothalamic regions involved in the regulation of food intake and energy balance, particularly in the paraventricular, ventromedial and arcuate nuclei (Ahima and Flier, 2000; Mercer *et al*., 1996). The extreme appetite and obesity of the *ob/ob* mouse is recapitulated by a mutation in the receptor gene resulting in complete absence of Ob-Rb [e.g., *db/db* mouse] (Chen *et al*., 1996; Lee *et al*., 1996). A similar phenotype is seen with ablation of Ob-Rb in the central nervous system or loss of Ob-Rb mediated STAT3 activation (Bates *et al*., 2003; Cohen *et al*., 2001). Therefore, leptin acts predominantly via centrally located Ob-Rb receptors.

In rodents, four other receptor isoforms have intracellular domains of 40 amino acids or less (Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Rf) and lack the intracellular domain required for STAT3 activation (Ahima and Flier, 2000; Myers, 2004). Ob-Ra, the prototypical member of this subclass is nevertheless capable of weakly activating JAK2, IRS-1, and MAPK (Bjorbaek *et al*., 1997). Most peripheral tissues express at least one of these isoforms in far greater abundance than the Ob-Rb isoform (Ahima and Flier, 2000; Ghilardi *et al*., 1996). Finally, a last isoform (Ob-Re) contains only the extracellular binding domain (Gavrilova *et al*., 1997; Kratzsch *et al*., 2002). This isoform is generated by proteolytic cleavage of membrane bound receptors in most species (Ge *et al*., 2002; Maamra *et al*., 2001) and by translation of a specific mRNA in the mouse (Gavrilova *et al*.,

1997). It acts as a specific leptin binding protein in plasma and is often referred to as the soluble leptin receptor.

In cattle and sheep, the various transcripts produced by the leptin receptor gene have not been described. Distribution of the leptin receptor has been deduced by *in situ* hybridization using partial cDNAs covering a portion of the extracellular region of the leptin receptor or intracellular regions specific for Ob-Rb (Laud *et al*., 1999; Williams *et al*., 1999). As in rodents, Ob-Rb expression was located to various brain regions and more importantly to the paraventricular, ventromedial and arcuate nuclei of the hypothalamus (Williams *et al*., 1999). Clarke and colleagues extended these results by showing that Ob-Rb immunoreactivity was present in these hypothalamic areas (Iqbal *et al*., 2001).

More recently, the presence of Ob-Ra and Ob-Rb was examined in many peripheral bovine tissues by non-quantitative RT-PCR. In contrast to the preponderance of data in other species, the Ob-Rb transcript was found ubiquitously whereas the Ob-Ra transcript was restricted to five out of the twenty-seven tissues surveyed (Chelikani *et al*., 2003a; Silva *et al*., 2002). In the case of Ob-Rb, these results likely reflect the ability of this technique to produce strong signals despite nearly meaningless mRNA levels. These data do not exclude the possibility that short receptor isoforms other than Ob-Ra predominate in most peripheral tissues. For example, both short and long forms of the receptor were detected by RT-PCR in the developing ovine mammary gland, but only the short isoforms were detected by ribonuclease protection assays, indicating that they accounted for most of the leptin receptor mRNA (Laud *et al*., 1999). Peripheral leptin actions certainly occur in ruminants, but they are likely restricted to sub-populations of cells expressing significant levels of the Ob-Rb isoform, such as somatotropes and gonadotropes (Amstalden *et al*., 2003; Iqbal *et al*., 2000; Roh *et al*., 2001).

Regulation of plasma leptin in dairy cows

Using RIAs that work equally well in all ruminants, plasma leptin was shown to be positively regulated by fatness and to a lesser extent by the plane of nutrition in non-lactating sheep and cattle (Blache *et al*., 2000b; Block *et al*., 2003b; Delavaud *et al*., 2000; Delavaud *et al*., 2002; Ehrhardt *et al*., 2000). Similar regulation occurs in dairy cattle at all stages of the lactation cycle (non-pregnant non-lactating, late pregnant and early lactating animals), but the effects of adiposity appear attenuated during lactation (Block *et al*., 2003a; Liefers *et al*., 2003b). A suppressive effect of lactation has also been observed in dairy goats (Bonnet *et al*., 2005). Unlike rodents and humans (Ahima and Flier, 2000), sheep and dairy cattle do not show diurnal changes in plasma leptin concentrations (Blache *et al*., 2000b; Block *et al*., 2001).

A number of studies have looked at the regulation of plasma leptin in high yielding dairy cows during the transition from pregnancy to lactation. The plasma concentration of leptin is highest during pregnancy and is reduced by 25-50% during the few days surrounding parturition (Block *et al*., 2001; Holtenius *et al*., 2003; Liefers *et al*., 2003b; Reist *et al*., 2003). This rapid reduction in plasma leptin was shown to be temporally associated with a reduction in leptin gene expression in subcutaneous adipose tissue and was attributed to the onset of negative energy balance (Block *et al*., 2001). Reduction in plasma leptin in early lactation is partly explained by loss of placental

synthesis of leptin in humans (Masuzaki *et al*., 1997) or the soluble receptor in the mouse (Gavrilova *et al*., 1997). These factors are negligible in ruminants. Leptin is not expressed in the ovine placenta at any stage of gestation (Ehrhardt *et al*., 2002). Secondly, leptin binding activity is slighly higher in late pregnancy than in early lactation in dairy cattle plasma (Figure 1), but GH competes this binding as effectively as leptin, indicating that the soluble leptin receptor does not account for this phenomenon. These conclusions were formally proven by demonstrating that plasma leptin remains elevated after parturition when negative energy balance is prevented by cessation of milk synthesis (Block *et al*., 2001).

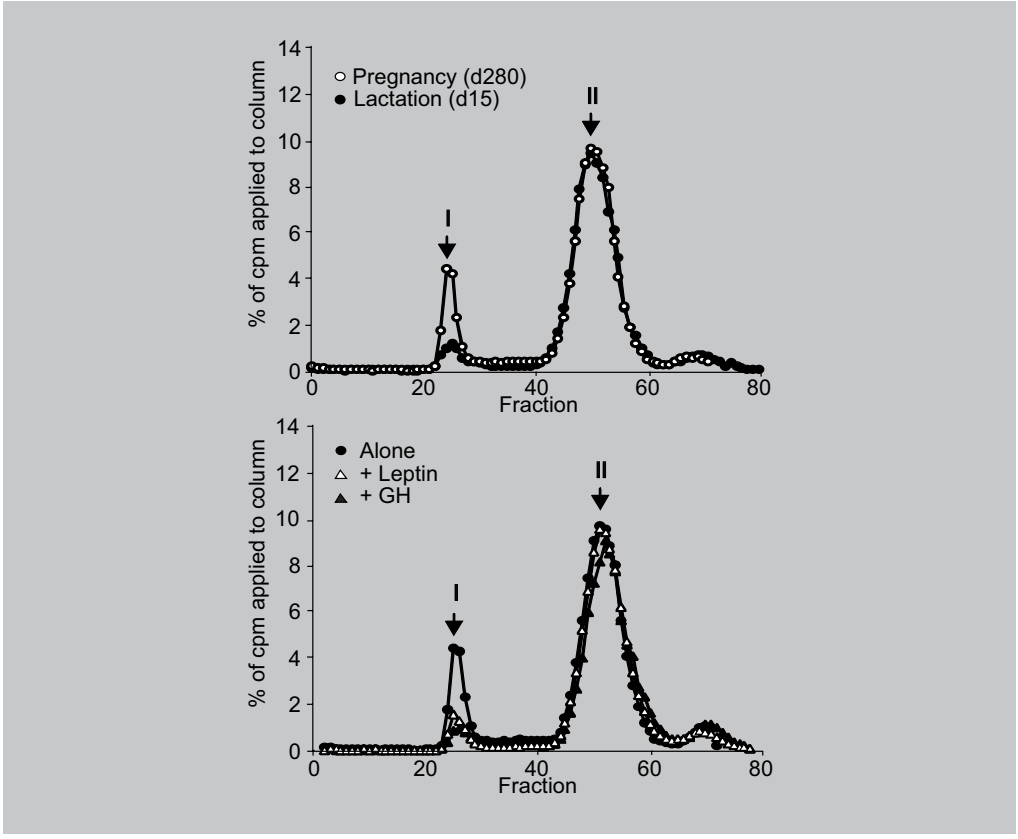

Figure 1. Analysis of bovine plasma for the presence of leptin binding protein. Plasma was obtained from dairy cows at day 280 of pregnancy (pregnancy) and at day 15 of lactation (lactation). Data from one representative cow are shown in both panels. Top panel: One hundred microliters of plasma were incubated for 30 min at 37 with ^{125}I labeled recombinant bovine leptin. Samples were separated by gel filtration chromatography on a Sephadex G150 column and eluted with buffer (10 mM Tris, 150 mM NaCl; pH=8.00). Peaks I and II represents bound and free leptin eluting at >120 kDa and 16 kDa, respectively. Bottom panel: The gel filtration analysis was repeated with pregnancy plasma incubated alone or in the presence of 10 μg unlabeled recombinant bovine leptin or growth hormone (GH).

The periparturient reduction in plasma leptin coincides with decreased plasma insulin (Block *et al*., 2001), a hormone shown to stimulate leptin synthesis in rodents and humans (Ahima and Flier, 2000; Ingvartsen and Boisclair, 2001). These observations suggested that reduced plasma leptin could represent decreased stimulation by insulin. Consistent with this, hyperinsulinemic-euglycemic clamps increased circulating leptin at all stages of the pregnancy-lactation cycle (Block *et al*., 2003a; Leury *et al*., 2003). The increased plasma GH of early lactation could also be involved in the fall of plasma leptin given its ability to antagonize insulin actions in adipose tissue (Bauman, 2000; Etherton and Bauman, 1998). However, chronic GH treatment did not reduce plasma leptin during lactation, but apparently inhibited insulin-mediated leptin synthesis in late pregnancy (Block *et al*., 2003a; Leury *et al*., 2003). These data are consistent with *in vitro* results showing that GH attenuates the positive effects of insulin on leptin synthesis in adipose tissue explants, but has little effect on its own (Houseknecht *et al*., 2000). Finally, β-adrenergic signaling inhibits leptin secretion in rodent adipocytes, and a similar effect is seen in cattle (Chilliard *et al*., 2001). Therefore, it is likely that increased β-adrenergic responsiveness of adipose tissue in early lactation also contributes to reduced leptin synthesis.

In dairy cows, the post-parturient depression in plasma leptin persists during early lactation despite a gradual improvement in energy balance (Block *et al*., 2001; Liefers *et al*., 2003b). This is likely related to depletion of white adipose tissue in high yielding dairy cows. Over time, plasma leptin increases as body reserves are replenished (Block *et al*., 2003a; Chelikani *et al*., 2003b), but this increase is small suggesting a suppressive effect of lactation. Consistent with this, insulin caused a smaller plasma leptin response during lactation than during late pregnancy (Block *et al*., 2003a; Leury *et al*., 2003).

Possible consequences of decreased plasma leptin in early lactating ruminants

At this point, we do not know whether the periparturient fall in plasma leptin has functional consequences in early lactating ruminants. The functional significance of this fall can only be inferred from experiments performed in non-lactating animals (peripubertal beef heifers, ovarectomized ewes or cows, oestradiol-implanted castrated or intact rams). Leptin exerts potent satiety effects on the ruminant brain as shown by the consistent reduction in feed intake caused by chronic intracerebroventricular infusion of leptin (Blache *et al*., 2000a; Henry *et al*., 1999; Miller *et al*., 2002; Morrison *et al*., 2001). This reduction is seen within a day and can reach > 75% after 3-5 days of infusion. As stated in the introduction, the energy deficit of early lactation results from inadequate voluntary feed intake after parturition. Therefore, reduced plasma leptin around parturition could facilitate a faster increase in voluntary feed intake, particularly if late pregnancy and early lactation are not leptin-resistant states.

In cattle, evidence based on positive associations between missense mutations in the leptin gene and performance suggests the possibility that variation in bioactive leptin impacts feed intake. Three such mutations have been reported in bovine leptin, changing tyrosine to phenylalanine at position 7 of the signal peptide, arginine to cysteine at position 4 of the mature peptide (R4C) or alanine to valine at position 59 of the mature peptide (Buchanan *et al*., 2002; Lagonigro *et al*., 2003; Liefers *et al*., 2003a; Liefers *et al*., 2002). The R4C mutation has received more attention because the additional cysteine has the potential to interfere with the critical disulfide bridge

between cysteines 96 and 146 of mature leptin. Homozygosity for this mutation resulted in fatter beef cattle in one study (Buchanan *et al*., 2002) and in increased milk yield in early lactating dairy cattle in a second study (Buchanan *et al*., 2003). The simplest explanation for these effects is that the R4C leptin is less potent, and that the associated phenotypic effects result from increased feed intake. However, formal demonstration that the R4C leptin is a weaker agonist or that homozygous R4C animals have higher feed intake, is missing. Moreover, others have failed to detect positive effects of this variant on various traits (dry matter intake, milk yield, energy balance and fatness) in dairy or beef cattle (Lagonigro *et al*., 2003; Liefers, 2004).

Flier and colleagues have argued that the primary role of leptin is to signal nutritional adequacy (Ahima and Flier, 2000; Spiegelman and Flier, 2001). According to this model, a threshold level of plasma leptin is needed to maintain neuro-endocrine functions responsible for processes that are not absolutely essential for short-term survival such as reproduction. When plasma leptin falls below this threshold, the secretion of neurally regulated hormones such as luteinizing hormone (LH) and the associated reproductive functions are impaired. This concept is supported by the ability of leptin to accelerate the resumption of full ovarian cycle after fasting in mice (Ahima *et al*., 1996). Early lactating dairy cows often suffer from delayed or failed first ovulation and decreased fertility (Butler, 2000; Lucy, 2001). These defects have been attributed to the ability of negative energy balance to suppress pulsatile LH secretion, a process necessary for ovulation of the dominant ovarian follicle. Leptin administration rescues LH pulsatility in fasted oestradiol-treated castrated rams and in peripubertal beef heifers (Maciel *et al*., 2004; Nagatani *et al*., 2000). Leptin also stimulates other LH secretory characteristics in mature ovarectomized ewes and beef cows when fasted or chronically undernourished, but not when well-fed (Amstalden *et al*., 2002; Henry *et al*., 1999; Henry *et al*., 2001). Overall, these data suggest that the hypoleptinemia of early lactation could contribute to the impaired reproductive activity of early lactation. Consistent with this possibility, positive associations were reported in postparturient dairy cows between the interval from parturition to the leptin nadir and the time of first ovulation (Kadokawa *et al*., 2000), and between plasma leptin and time of first estrus (Liefers *et al*., 2003b). Whether leptin treatment of early lactating dairy cows can stimulate LH pulsation, first ovulation and overall fertility remains to be demonstrated.

As discussed in the first section, GH orchestrates peripheral metabolic adaptations during periods of nutritional insufficiency, and is also secreted in response to neuro-peptides (i.e, GH releasing hormone and somatostatin). If leptin serves as a signal of energy sufficiency on the GH axis, leptin should reverse changes in GH secretion caused by caloric deprivation. These effects of leptin should be opposite in rodents and ruminants because undernutrition decreases GH secretion in the former, but increases it in the latter (Thissen *et al*., 1999). Consistent with this prediction, leptin stimulated GH secretion in rodents consuming inadequate amounts of calories or food deprived (Carro *et al*., 1997; Tannenbaum *et al*., 1998), but prevented the increase seen in pair-fed intact rams and in fasted overectomized adult ewes (Blache *et al*., 2000a; Henry *et al*., 2004). However, both central and peripheral leptin treatment caused paradoxical increases in GH secretion in other studies involving well-fed, fasted or chronically undernourished ruminants (Garcia *et al*., 2002; Henry *et al*., 2001; Nagatani *et al*., 2000). This inconsistency is not explained by the mode of leptin administration because central administration increased GH secretion in freely feeding animals in one experiment (Henry *et al*., 2001) and decreased it in others (Blache *et*

al., 2000a; Henry *et al*., 2004). Variation in GH response could represent interactions between the model used (gonadectomized *vs* intact, male vs female, etc), prevailing adiposity and the nature of caloric restriction (severity and length) (Henry *et al*., 2004).

Conclusions

Bauman and Vernon proposed that GH actions are reduced in liver during periods of severe undernutrition such as the periparturient period, limiting IGF-I production and its associated anabolic effects (Bauman and Vernon, 1993). They also proposed that GH contributes to the maintenance of metabolic homeostasis by remaining fully effective in skeletal muscle and adipose tissue. The recent data presented on GHR regulation in this review adds mechanistic details to this model, but many questions remain. The mechanisms responsible for the reduction of GHR1A mRNA in the periparturient cow remain poorly defined. It is also unlikely that GHR variation alone completely explains changes in GH responsiveness. Indeed, in rodents, defects in post-receptor signaling account for loss of GH actions in liver and other tissues in a variety of conditions (Beauloye *et al*., 2002; Mao *et al*., 1999; Schaefer *et al*., 2001). Finally, aside from decreased hepatic IGF-I production, we still do not know what the functional consequences of variation in GHR abundance in liver and non-hepatic tissues are in early lactation.

In rodents, leptin is reduced in undernourished animals, and direct experimental evidence indicates that this deficit triggers neuro-endocrine adaptations responsible for energy conservation and metabolic coordination (Ahima and Flier, 2000). Therefore, the regulation and actions of leptin in rodents are generally consistent with a homeorhetic role. In dairy cattle, changes in plasma leptin during periods of nutritional insufficiency such as the transition from pregnancy to lactation also fits such a role (Block *et al*., 2001; Holtenius *et al*., 2003; Reist *et al*., 2003). Leptin is a conceptually attractive candidate because the focus of its action is the brain, and therefore could work in concert with GH, which acts almost exclusively on peripheral tissues. The challenge in the next few years will be to provide experimental proof for this hypothesis in ruminants. Peculiarities of the leptin system will need to be considered in designing these experiments and in interpreting the results. For instance, less leptin, not more, is the biologically relevant signal perceived by the brain (Ahima and Flier, 2000). Moreover, data obtained in rodents suggest that the fully effective dose of leptin is low for many biological responses, and therefore the ability of exogenous administration of leptin to reveal important leptin actions may be limited.

Acknowledgements

This work was supported by the Cornell University Agricultural Research Station and by grants from the National Research Initiative Competitive Research Program (USDA CRESS 00-35206-9352 and 02-35203-12832).

References

Ahima, R.S. & J.S. Flier (2000). Leptin. Annual Review Physiolology. 62:413-437.

Ahima, R.S., D. Prabakaran, C. Mantzoros, D. Qu, B. Lowell, E. Maratos-Flier & J.S. Flier (1996). Role of leptin in the neuroendocrine response to fasting. Nature. 382:250-252.

Akers, R.M. (1985). Lactogenic hormones: binding sites, mammary growth, secretory cell differentiation, and milk biosynthesis in ruminants. Journal of Dairy Science. 68:501-519.

Amstalden, M., M.R. Garcia, R.L. Stanko, S.E. Nizielski, C.D. Morrison, D.H. Keisler & G.L. Williams (2002). Central infusion of recombinant ovine leptin normalizes plasma insulin and stimulates a novel hypersecretion of luteinizing hormone after short-term fasting in mature beef cows. Biology of Reproduction. 66:1555-1561.

Amstalden, M., D.A. Zieba, J.F. Edwards, P.G. Harms, T.H. Welsh, Jr., R.L. Stanko & G.L. Williams (2003). Leptin acts at the bovine adenohypophysis to enhance basal and gonadotropin-releasing hormone-mediated release of luteinizing hormone: differential effects are dependent upon nutritional history. Biology of Reproduction. 69:1539-1544.

Bates, S.H., W.H. Stearns, T.A. Dundon, M. Schubert, A.W. Tso, Y. Wang, A.S. Banks, H.J. Lavery, A.K. Haq, E. Maratos-Flier, B.G. Neel, M.W. Schwartz & M.G. Myers, Jr. (2003). STAT3 signalling is required for leptin regulation of energy balance but not reproduction. Nature. 421:856-859.

Bauman, D.E. (1999). Bovine somatotropin and lactation: from basic science to commercial application. Domestic Animal Endocrinology. 17:101-116.

Bauman, D.E. (2000). Regulation of nutrient partitioning during lactation: Homeostasis and homeorhesis revisited. Pages 311-328 *in* Ruminant Physiology, Digestion, Metabolism, Growth and Reproduction. Proceedings 9th International Symposium on Ruminant Physiology. P.B. Cronje, ed. Commonwealth Agricultural Bureau International.

Bauman, D.E. & W.B. Currie (1980). Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homeostasis and homeorhesis. Journal of Dairy Science. 63:1514-1529.

Bauman, D.E., C.J. Peel, W.D. Steinhour, P.J. Reynolds, H.F. Tyrrell, A.C. Brown & G.L. Haaland (1988). Effect of bovine somatotropin on metabolism of lactating dairy cows: influence on rates of irreversible loss and oxidation of glucose and nonesterified fatty acids. Journal of Nutrition. 118:1031-1040.

Bauman, D.E. & R.G. Vernon (1993). Effects of exogenous bovine somatotropin on lactation. Annual Review of Nutrition. 13:437-461.

Beauloye, V., B. Willems, V. de Coninck, S.J. Frank, M. Edery & J.P. Thissen (2002). Impairment of liver GH receptor signaling by fasting. Endocrinology. 143:792-800.

Bell, A.W. (1995). Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of Animal Science. 73:2804-2819.

Bell, A.W. & D.E. Bauman (1997). Adaptations of glucose metabolism during pregnancy and lactation. Journal of Mammary Gland Biology and Neoplasia. 2:265-278.

Bjorbaek, C., S. Uotani, B. da Silva & J.S. Flier (1997). Divergent signaling capacities of the long and short isoforms of the leptin receptor. Journal of Biological Chemistry. 272:32686-32695.

Blache, D., P. Celi, M.A. Blackberrv, R.A. Dynes & G.B. Martin (2000a). Decrease in voluntary feed intake and pulsatile luteinizing hormone secretion after intracerebroventricular infusion of recombinant bovine leptin in mature male sheep. Reproduction, Fertility and Development. 12:373-381.

Blache, D., R.L. Tellam, L.M. Chagas, M.A. Blackberry, P.E. Vercoe & G.B. Martin (2000b). Level of nutrition affects leptin concentrations in plasma and cerebrospinal fluid in sheep. Journal of Endocrinology. 165:625-637.

Block, S.S., W.R. Butler, R.A. Ehrhardt, A.W. Bell, M.E. Van Amburgh & Y.R. Boisclair (2001). Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. Journal of Endocrinology. 171:339-348.

Block, S.S., R.P. Rhoads, D.E. Bauman, R.A. Ehrhardt, M.A. McGuire, B.A. Crooker, J.M. Griinari, T.R. Mackle, W.J. Weber, M.E. Van Amburgh & Y.R. Boisclair (2003a). Demonstration of a role for insulin in the regulation of leptin in lactating dairy cows. Journal of Dairy Science. 86:3508-3515.

Block, S.S., J.M. Smith, R.A. Ehrhardt, M.C. Diaz, R.P. Rhoads, M.E. Van Amburgh & Y.R. Boisclair (2003b). Nutritional and developmental regulation of plasma leptin in dairy cattle. Journal of Dairy Science. 86:3206-3214.

Bonnet, M., I. Gourdou, C. Leroux, Y. Chilliard & J. Djiane (2002). Leptin expression in the ovine mammary gland: putative sequential involvement of adipose, epithelial, and myoepithelial cells during pregnancy and lactation. Journal of Animal Science. 80:723-728.

Bonnet, M., C. Delavaud, J. Rouel & Y. Chilliard (2005). Pregnancy increases plasma leptin in nulliparous but not primiparous goats while lactation depresses it. Domestic Animal Endocrinology, 28:216-223.

Buchanan, F.C., C.J. Fitzsimmons, A.G. Van Kessel, T.D. Thue, D.C. Winkelman-Sim & S.M. Schmutz (2002). Association of a missense mutation in the bovine leptin gene with carcass fat content and leptin mRNA levels. Genetics, Selection, Evolution. 34:105-116.

Buchanan, F.C., A.G. Van Kessel, C. Waldner, D.A. Christensen, B. Laarveld & S.M. Schmutz (2003). Hot topic: an association between a leptin single nucleotide polymorphism and milk and protein yield. Journal of Dairy Science. 86:3164-3166.

Butler, S.T., A.L. Marr, S.H. Pelton, R.P. Radcliff, M.C. Lucy & W.R. Butler (2003). Insulin restores GH responsiveness during lactation-induced negative energy balance in dairy cattle: effects on expression of IGF-I and GH receptor 1A. Journal of Endocrinology. 176:205-217.

Butler, W.R. (2000). Nutritional interactions with reproductive performance in dairy cattle. Animal Reproduction Science. 60-61:449-457.

Caro, J.F., J. Poulos, O. Ittoop, W.J. Pories, E.G. Flickinger & M.K. Sinha (1988). Insulin-like growth factor I binding in hepatocytes from human liver, human hepatoma, and normal, regenerating, and fetal rat liver. The Journal of Clinical Investigation. 81:976-981.

Carro, E., R. Senaris, R.V. Considine, F.F. Casanueva & C. Dieguez (1997). Regulation of in vivo growth hormone secretion by leptin. Endocrinology. 138:2203-2206.

Chehab, F.F., M.E. Lim & R. Lu (1996). Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. Nature Genetics. 12:318-320.

Chelikani, P.K., D.R. Glimm & J.J. Kennelly (2003a). Short communication: Tissue distribution of leptin and leptin receptor mRNA in the bovine. Journal of Dairy Science. 86:2369-2372.

Chelikani, P.K., D.H. Keisler & J.J. Kennelly (2003b). Response of plasma leptin concentration to jugular infusion of glucose or lipid is dependent on the stage of lactation of Holstein cows. Journal of Nutrition. 133:4163-4171.

Chen, H., O. Charlat, L.A. Tartaglia, E.A. Woolf, X. Weng, S.J. Ellis, D.N. Lakey, J. Culpepper, K.J. Moore, R.D. Breitbart, G.M. Duyk, R.I. Tepper & J.P. Morgenstern (1996). Evidence that the diabetes gene encodes the leptin receptor: Identification of a mutation in the leptin receptor gene in *db/db* mice. Cell. 84:491-495.

Chilliard, Y., M. Bonnet, C. Delavaud, Y. Faulconnier, C. Leroux, J. Djiane & F. Bocquier (2001). Leptin in ruminants. Gene expression in adipose tissue and mammary gland, and regulation of plasma concentration. Domestic Animal Endocrinology. 21:271-295.

Cohen, P., C. Zhao, X. Cai, J.M. Montez, S.C. Rohani, P. Feinstein, P. Mombaerts & J.M. Friedman (2001). Selective deletion of leptin receptor in neurons leads to obesity. The Journal of Clinical Investigation. 108:1113-1121.

Collier, R.J., M.F. McGrath, J.C. Byatt & L.L. Zurfluh (1993). Regulation of bovine mammary growth by peptide hormones: involvement of receptors, growth factors and binding proteins. Livestock Production Science. 35:21-33.

Dehoff, M.H., R.G. Elgin, R.J. Collier & D.R. Clemmons (1988). Both type I and II insulin-like growth factor receptor binding increase during lactogenesis in bovine mammary tissue. Endocrinology. 122:2412-2417.

Delavaud, C., F. Bocquier, Y. Chilliard, D.H. Keisler, A. Gertler & G. Kann (2000). Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA in sheep. Journal of Endocrinology. 165:519-526.

Delavaud, C., A. Ferlay, Y. Faulconnier, F. Bocquier, G. Kann & Y. Chilliard (2002). Plasma leptin concentration in adult cattle: effects of breed, adiposity, feeding level, and meal intake. Journal of Animal Science. 80:1317-1328.

Drackley, J.K., T.R. Overton & G.N. Douglas (2001). Adaptations of glucose and long-chain fatty acid metabolism in liver of dairy cows during the periparturient period. Journal of Dairy Science. 84:E100-E112. [This paper is available at http://www.adsa.org/jds/abs/2001/jds_es2100.htm].

Dyer, C.J., J.M. Simmons, R.L. Matteri & D.H. Keisler (1997). cDNA cloning and tissue-specific gene expression of ovine leptin, NYP-Y1 receptor, and NYP-Y2 receptor. Domestic Animal Endocrinology. 14:295-303.

Ehrhardt, R.A., A.W. Bell & Y.R. Boisclair (2002). Spatial and developmental regulation of leptin in fetal sheep. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 282: R1628-1635.

Ehrhardt, R.A., R.M. Slepetis, M.E. VanAmburgh, J. Siegal-Willot, A.W. Bell & Y.R. Boisclair (2000). Development of a specific RIA to measure physiological changes of circulating leptin in cattle and sheep. Journal of Endocrinology. 166:519-528.

Etherton, T.D. & D.E. Bauman (1998). Biology of somatotropin in growth and lactation of domestic animals. Physiological Reviews. 78:745-761.

Fryburg, D.A., L.A. Jahn, S.A. Hill, D.M. Oliveras & E.J. Barrett (1995). Insulin and insulin-like growth factor-I enhance human skeletal muscle protein anabolism during hyperaminoacidemia by different mechanisms. The Journal of Clinical Investigation. 96:1722-1729.

Garcia, M.R., M. Amstalden, S.W. Williams, R.L. Stanko, C.D. Morrison, D.H. Keisler, S.E. Nizielski & G.L. Williams (2002). Serum leptin and its adipose gene expression during pubertal development, the estrous cycle, and different seasons in cattle. Journal of Animal Science. 80:2158-2167.

Gavrilova, O., V. Barr, B. Marcus-Samuels & M. Reitman (1997). Hyperleptinemia of pregnancy associated with the appearance of a circulating form of the leptin receptor. Journal of Biological Chemistry. 272:30546-30551.

Ge, H., L. Huang, T. Pourbahrami & C. Li (2002). Generation of soluble leptin receptor by ectodomain shedding of membrane-spanning receptors in vitro and in vivo. Journal of Biological Chemistry. 277:45898-45903.

Ghilardi, N., S. Ziegler, A. Wiestner, R. Stoffel, M.H. Heim & R.C. Skoda (1996). Defective STAT signaling by the leptin receptor in diabetic mice. Proceedings of the National Academy of Sciences of the United States of America. 93:6231-6235.

Goff, J.P. & R.L. Horst (1997). Physiological changes at parturition and their relationship to metabolic disorders. Journal of Dairy Science. 80:1260-1268.

Hadsell, D.L., S.G. Bonnette & A.V. Lee (2002). Genetic manipulation of the IGF-I axis to regulate mammary gland development and function. Journal of Dairy Science. 85:365-377.

Halaas, J.L., K.S. Gajiwala, M. Maffei, S.L. Cohen, B.T. Chait, D. Rabinowitz, R.L. Lallone, S.K. Burley & J.M. Friedman (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. Science. 269:543-546.

Henry, B.A., J.W. Goding, W.S. Alexander, A.J. Tilbrook, B.J. Canny, F. Dunshea, A. Rao, A. Mansell & I.J. Clarke (1999). Central administration of leptin to ovariectomized ewes inhibits food intake without affecting the secretion of hormones from the pituitary gland: Evidence for a dissociation of effects on appetite and neuroendocrine function. Endocrinology. 140:1175-1182.

Henry, B.A., J.W. Goding, A.J. Tilbrook, F.R. Dunshea, D. Blache & I.J. Clarke (2004). Leptin-mediated effects of undernutrition or fasting on luteinizing hormone and growth hormone secretion in ovariectomized ewes depend on the duration of metabolic perturbation. Journal of Neuroendocrinology. 16:244-255.

Henry, B.A., J.W. Goding, A.J. Tilbrook, F.R. Dunshea & I.J. Clarke (2001). Intracerebroventricular infusion of leptin elevates the secretion of luteinising hormone without affecting food intake in long-term food-restricted sheep, but increases growth hormone irrespective of bodyweight. Journal of Endocrinology. 168:67-77.

Holtenius, K., S. Agenas, C. Delavaud & Y. Chilliard (2003). Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. Journal of Dairy Science. 86:883-891.

Houseknecht, K.L., C.P. Portocarrero, S. Ji, R. Lemenager & M.E. Spurlock (2000). Growth hormone regulates leptin gene expression in bovine adipose tissue: correlation with adipose IGF-1 expression. Journal of Endocrinology. 164:51-57.

Ingvartsen, K.L. & Y.R. Boisclair (2001). Leptin and the regulation of food intake, energy homeostasis and immunity with special focus on periparturient ruminants. Domestic Animal Endocrinology. 21:215-250.

Ingvartsen, K.L., R.J. Dewhurst & N.C. Friggens (2003). On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. Livest Prod Sci. 83:277-308.

Iqbal, J., S. Pompolo, R.V. Considine & I.J. Clarke (2000). Localization of leptin receptor-like immunoreactivity in the corticotropes, somatotropes, and gonadotropes in the ovine anterior pituitary. Endocrinology. 141:1515-1520.

Iqbal, J., S. Pompolo, T. Murakami, E. Grouzmann, T. Sakurai, B. Meister & I.J. Clarke (2001). Immunohistochemical characterization of localization of long-form leptin receptor (OB-Rb) in neurochemically defined cells in the ovine hypothalamus. Brain Research. 920:55-64.

Ji, S., G.M. Willis, R.R. Scott & M.E. Spurlock (1998). Partial cloning and expression of the bovine leptin gene. Animal Biotechnology. 9:1-14.

Jiang, H. & M.C. Lucy (2001a). Involvement of hepatocyte nuclear factor-4 in the expression of the growth hormone receptor 1A messenger ribonucleic acid in bovine liver. Molecular Endocrinology. 15:1023-1034.

Jiang, H. & M.C. Lucy (2001b). Variants of the 5'-untranslated region of the bovine growth hormone receptor mRNA: isolation, expression and effects on translational efficiency. Gene. 265:45-53.

Jiang, H., M.C. Lucy, B.A. Crooker & W.E. Beal (2005). Expression of growth hormone receptor 1A mRNA is decreased in dairy cows but not in beef cows at parturition. Journal of Dairy Science. 88:1370-1377..

Jiang, H., C.S. Okamura, C.K. Boyd & M.C. Lucy (2000). Identification of Sp1 as the transcription factor for the alternative promoter P2 of the bovine growth hormone receptor gene. Journal of Molecular Endocrinology. 24:203-214.

Jiang, H., C.S. Okamura & M.C. Lucy (1999). Isolation and characterization of a novel promoter for the bovine growth hormone receptor gene. Journal of Biological Chemistry. 274:7893-7900.

Kadokawa, H., D. Blache, Y. Yamada & G.B. Martin (2000). Relationships between changes in plasma concentrations of leptin before and after parturition and the timing of first post-partum ovulation in high-producing Holstein dairy cows. Reproduction, Fertility and Development. 12:405-411.

Kim, J.W., R.P. Rhoads, S.S. Block, T.R. Overton, S.J. Frank & Y.R. Boisclair (2004). Dairy cows experience selective reduction of the hepatic growth hormone receptor during the periparturient period. Journal of Endocrinology. 181:281-290.

Kobayashi, Y., C.K. Boyd, C.J. Bracken, W.R. Lamberson, D.H. Keisler & M.C. Lucy (1999a). Reduced growth hormone receptor (GHR) messenger ribonucleic acid in liver of periparturient cattle is caused by a specific down-regulation of GHR 1A that is associated with decreased insulin-like growth factor I. Endocrinology. 140:3947-3954.

Kobayashi, Y., C.K. Boyd, C.J. Bracken, W.R. Lamberson & M.C. Lucy (1999b). Expression of growth hormone receptor (GHR)1A and IGF-I mRNA after administration of estradiol 17β or glucocorticoid (G) in cattle. Journal of Dairy Science. 82(Suppl. 1):49 (Abstr.).

Kobayashi, Y., C.K. Boyd, B.L. McCormack & M.C. Lucy (2002). Reduced insulin-like growth factor-I after acute feed restriction in lactating dairy cows is independent of changes in growth hormone receptor 1A mRNA. Journal of Dairy Science. 85:748-754.

Kratzsch, J., A. Lammert, A. Bottner, B. Seidel, G. Mueller, J. Thiery, J. Hebebrand & W. Kiess (2002). Circulating soluble leptin receptor and free leptin index during childhood, puberty, and adolescence. Journal of Clinical Endocrinology and Metabolism. 87:4587-4594.

Lagonigro, R., P. Wiener, F. Pilla, J.A. Woolliams & J.L. Williams (2003). A new mutation in the coding region of the bovine leptin gene associated with feed intake. Animal Genetics. 34:371-374.

Laud, K., I. Gourdou, L. Belair, D.H. Keisler & J. Djiane (1999). Detection and regulation of leptin receptor mRNA in ovine mammary epithelial cells during pregnancy and lactation. FEBS Letters. 463:194-198.

Le Roith, D., C. Bondy, S. Yakar, J.L. Liu & A. Butler (2001). The somatomedin hypothesis: 2001. Endocrine Reviews. 22:53-74.

Lee, G.-H., R. Proenca, J.M. Montez, K.M. Carroll, J.G. Darvishzadeth, J.I. Lee & J.M. Friedman (1996). Abnormal splicing of the leptin receptor in diabetic mice. Nature. 379:632-635.

Leury, B.J., L.H. Baumgard, S.S. Block, N. Segoale, R.A. Ehrhardt, R.P. Rhoads, D.E. Bauman, A.W. Bell & Y.R. Boisclair (2003). Effect of insulin and growth hormone on plasma leptin in periparturient dairy cows. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 285:R1107-1115.

Liefers, S.C. (2004). Physiology and genetics of leptin in periparturient dairy cows. Ph.D., Wageningen University, Wageningen.

Liefers, S.C., M.F. te Pas, R.F. Veerkamp, Y. Chilliard, C. Delavaud, R. Gerritsen & T. van der Lende (2003a). Association of leptin gene polymorphisms with serum leptin concentration in dairy cows. Mammalian Genome. 14:657-663.

Liefers, S.C., M.F. te Pas, R.F. Veerkamp & T. van der Lende (2002). Associations between leptin gene polymorphisms and production, live weight, energy balance, feed intake, and fertility in Holstein heifers. Journal of Dairy Science. 85:1633-1638.

Liefers, S.C., R.F. Veerkamp, M.F. te Pas, C. Delavaud, Y. Chilliard & T. van der Lende (2003b). Leptin concentrations in relation to energy balance, milk yield, intake, live weight, and estrus in dairy cows. Journal of Dairy Science. 86:799-807.

Lord, G.M., G. Matarese, J.K. Howard, R.J. Baker, S.R. Bloom & R.I. Lechler (1998). Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature. 394:897-900.

Lucy, M.C. (2001). Reproductive loss in high-producing dairy cattle: where will it end? Journal of Dairy Science. 84:1277-1293.

Lucy, M.C., H. Jiang & Y. Kobayashi (2001). Changes in the Somatotrophic Axis Associated with the Initiation of Lactation. Journal of Dairy Science. 84(E. Suppl.):E113-119. [This paper is available at www.adsa.org/jds/abs/2001/jds_es2113.htm].

Maamra, M., M. Bidlingmaier, M.C. Postel-Vinay, Z. Wu, C.J. Strasburger & R.J. Ross (2001). Generation of human soluble leptin receptor by proteolytic cleavage of membrane-anchored receptors. Endocrinology. 142:4389-4393.

Maciel, M.N., D.A. Zieba, M. Amstalden, D.H. Keisler, J.P. Neves & G.L. Williams (2004). Leptin prevents fasting-mediated reductions in pulsatile secretion of luteinizing hormone and enhances its gonadotropin-releasing hormone-mediated release in heifers. Biology of Reproduction. 70:229-235.

Mao, Y., P.R. Ling, T.P. Fitzgibbons, K.C. McCowen, G.P. Frick, B.R. Bistrian & R.J. Smith (1999). Endotoxin-induced inhibition of growth hormone receptor signaling in rat liver in vivo. Endocrinology. 140:5505-5515.

Masuzaki, H., Y. Ogawa, N. Sagawa, K. Hosoda, T. Matsumoto, H. Mise, H. Nishimura, Y. Yoshimasa, I. Tanaka, T. Mori & K. Nakao (1997). Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. Nature Medicine. 3:1029-1033.

Mercer, J.G., N. Hoggard, L.M. Williams, C.B. Lawrence, L.T. Hannah & P. Trayhurn (1996). Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. FEBS Letters. 387:113-116.

Miller, D.W., P.A. Findlay, M.A. Morrison, N. Raver & C.L. Adam (2002). Seasonal and dose-dependent effects of intracerebroventricular leptin on lh secretion and appetite in sheep. Journal of Endocrinology. 175:395-404.

Morrison, C.D., J.A. Daniel, B.J. Holmberg, J. Djiane, N. Raver, A. Gertler & D.H. Keisler (2001). Central infusion of leptin into well-fed and undernourished ewe lambs: effects on feed intake and serum concentrations of growth hormone and luteinizing hormone. Journal of Endocrinology. 168:317-324.

Myers, M.G., Jr. (2004). Leptin receptor signaling and the regulation of mammalian physiology. Recent Progress in Hormone Research. 59:287-304.

Nagatani, S., Y. Zeng, D.H. Keisler, D.L. Foster & C.A. Jaffe (2000). Leptin regulates pulsatile luteinizing hormone and growth hormone secretion in the sheep. Endocrinology. 141:3965-3975.

Plath-Gabler, A., C. Gabler, F. Sinowatz, B. Berisha & D. Schams (2001). The expression of the IGF family and GH receptor in the bovine mammary gland. Journal of Endocrinology. 168:39-48.

Prosser, C.G., S.R. Davis, V.C. Farr, L.G. Moore & P.D. Gluckman (1994). Effects of close-arterial (external pudic) infusion of insulin-like growth factor-II on milk yield and mammary blood flow in lactating goats. Journal of Endocrinology. 142:93-99.

Radcliff, R.P., B.L. McCormack, B.A. Crooker & M.C. Lucy (2003a). Growth hormone (GH) binding and expression of GH receptor 1A mRNA in hepatic tissue of periparturient dairy cows. Journal of Dairy Science. 86:3933-3940.

Radcliff, R.P., B.L. McCormack, B.A. Crooker & M.C. Lucy (2003b). Plasma hormones and expression of growth hormone receptor and insulin-like growth factor-I mRNA in hepatic tissue of periparturient dairy cows. Journal of Dairy Science. 86:3920-3926.

Reist, M., D. Erdin, D. von Euw, K. Tschuemperlin, H. Leuenberger, C. Delavaud, Y. Chilliard, H.M. Hammon, N. Kuenzi & J.W. Blum (2003). Concentrate feeding strategy in lactating dairy cows: metabolic and endocrine changes with emphasis on leptin. Journal of Dairy Science. 86:1690-1706.

Rhoads, R.P., J.W. Kim, B.J. Leury, L.H. Baumgard, N. Segoale, S.J. Frank, D.E. Bauman & Y.R. Boisclair (2004). Insulin increases the abundance of the growth hormone receptor in liver and adipose tissue of periparturient dairy cows. Journal of Nutrition. 134:1020-1027.

Richard, A.L., S.N. McCutcheon & D.E. Bauman (1985). Responses of Dairy Cows to Exogenous Bovine Growth Hormone Administered During Early Lactation. Journal of Dairy Science. 68:2385-2389.

Roh, S.G., G.Y. Nie, K. Loneragan, A. Gertler & C. Chen (2001). Direct modification of somatotrope function by long-term leptin treatment of primary cultured ovine pituitary cells. Endocrinology. 142:5167-5171.

Ronge, H. & J.W. Blum (1989). Insulinlike growth factor I resposes to growth hormone in dry and lactating dairy cows. Journal of Animal Physiology and Animal Nutrition. 62:280-288.

Schaefer, F., Y. Chen, T. Tsao, P. Nouri & R. Rabkin (2001). Impaired JAK-STAT signal transduction contributes to growth hormone resistance in chronic uremia. The Journal of Clinical Investigation. 108:467-475.

Sechen, S.J., F.R. Dunshea & D.E. Bauman (1990). Somatotropin in lactating cows: Effect on response to epinephrine and insulin. American Journal of Physiology. 258:E582-E588.

Shamay, A., N. Cohen, M. Niwa & A. Gertler (1988). Effect of insulin-like growth factor I on deoxyribonucleic acid synthesis and galactopoiesis in bovine undifferentiated and lactating mammary tissue *in vitro*. Endocrinology. 123:804-809.

Silva, L.F., M.J. VandeHaar, M.S. Weber Nielsen & G.W. Smith (2002). Evidence for a local effect of leptin in bovine mammary gland. Journal of Dairy Science. 85:3277-3286.

Singleton, J.R. & E.L. Feldman (2001). Insulin-like growth factor-I in muscle metabolism and myotherapies. Neurobiology of Disease. 8:541-554.

Sinowatz, F., D. Schams, S. Kolle, A. Plath, D. Lincoln & M.J. Waters (2000). Cellular localisation of GH receptor in the bovine mammary gland during mammogenesis, lactation and involution. Journal of Endocrinology. 166:503-510.

Sjogren, K., J.-L. Liu, K. Blad, S. Skrtic, O. Vidal, V. Wallenius, D. LeRoith, J. Tornell, O.G.P. Isaksson, J.-L. Jansson & C. Ohlsson (1999). Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. Proceedings of the National Academy of Sciences of the United States of America. 96:7088-7092.

Spiegelman, B.M. & J.S. Flier (2001). Obesity and the regulation of energy balance. Cell. 104:531-543.

Tannenbaum, G.S., W. Gurd & M. Lapointe (1998). Leptin is a potent stimulator of spontaneous pulsatile growth hormone (GH) secretion and the GH response to GH-releasing hormone. Endocrinology. 139:3871-3875.

Thissen, J.P., L.E. Underwood & J.M. Ketelslegers (1999). Regulation of insulin-like growth factor-I in starvation and injury. Nutrition Reviews. 57:167-176.

Ueki, I., G.T. Ooi, M.L. Tremblay, K.R. Hurst, L.A. Bach & Y.R. Boisclair (2000). Inactivation of the acid labile subunit gene in mice results in mild retardation of postnatal growth despite profound disruptions in the circulating insulin-like growth factor system. Proceedings of the National Academy of Sciences of the United States of America. 97:6868-6873.

Vernon, R.G. (2000). Adipocyte studies: systems for investigating effects of growth hormone and other chronically acting hormones. Biochemical Society Transactions. 28:126-131.

Vernon, R.G. & D.J. Flint. (1989). Role of growth hormone in the regulation of adipocyte growth and function. Pages 57-71 *in* Biotechnology in Growth Regulation. R.B. Heap, C.G. Prosser, and E. Lamming, eds. Butterworth, London.

Vernon, R.G. & C.M. Pond (1997). Adaptations of maternal adipose tissue to lactation. Journal of Mammary Gland Biology and Neoplasia. 2:231-241.

Vicini, J.L., F.C. Buonomo, J.J. Veenhuizen, M.A. Miller, D.R. Clemmons & R.J. Collier (1991). Nutrient balance and stage of lactation affect responses of insulin, insulin-like growth factors I and II, and insulin-like growth factor-binding protein 2 to somatotropin administration in dairy cows. Journal of Nutrition. 121:1656-1664.

Williams, L.M., C.L. Adam, J.G. Mercer, K.M. Moar, D. Slater, L. Hunter, P.A. Findlay & N. Hoggard (1999). Leptin receptor and neuropeptide Y gene expression in the sheep brain. Journal of Neuroendocrinology. 11:165-169.

Xu, Q., N. Walther & H. Jiang (2004). Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and hepatocyte nuclear factor 4gamma (HNF-4gamma) and HNF-4alpha regulate the bovine growth hormone receptor 1A promoter through a common DNA element. Journal of Molecular Endocrinology. 32:947-961.

Yakar, S., J.-L. Liu, B. Stannard, A. Butler, D. Accili, B. Sauer & D. LeRoith (1999). Normal growth and development in the absence of hepatic insulin-like growth factor I. Proceedings of the National Academy of Sciences of the United States of America. 96:7324-7329.

Yakar, S., C.J. Rosen, W.G. Beamer, C.L. Ackert-Bicknell, Y. Wu, J.L. Liu, G.T. Ooi, J. Setser, J. Frystyk, Y.R. Boisclair & D. LeRoith (2002). Circulating levels of IGF-1 directly regulate bone growth and density. The Journal of Clinical Investigation. 110:771-781.

Yakar, S., J. Setser, H. Zhao, B. Stannard, M. Haluzik, V. Glatt, M.L. Bouxsein, J.J. Kopchick & D. LeRoith (2004). Inhibition of growth hormone action improves insulin sensitivity in liver IGF-1-deficient mice. The Journal of Clinical Investigation. 113:96-105.

Yonekura, S., K. Kitade, G. Furukawa, K. Takahashi, N. Katsumata, K. Katoh & Y. Obara (2002). Effects of aging and weaning on mRNA expression of leptin and CCK receptors in the calf rumen and abomasum. Domestic Animal Endocrinology. 22:25-35.

Zhang, F., M.B. Basinski, J.M. Beals, S.L. Briggs, L.M. Churgay, D.K. Clawson, R.D. DiMarchi, T.C. Furman, J.E. Hale, H.M. Hsiung, B.E. Schoner, D.P. Smith, X.Y. Zhang, J.P. Wery & R.W. Schevitz (1997). Crystal structure of the obese protein leptin-E100. Nature. 387:206-209.

Zhang, Y., R. Proenca, M. Maffei, M. Barone, L. Leopold & J.M. Friedman (1994). Positional cloning of the mouse *obese* gene and its human homologue. Nature. 372:425-432.

Part V: Lactation and reproduction physiology

Prenatal nutrition, fetal programming and opportunities for farm animal research

B.H. Breier
Liggins Institute for Medical Research and National Research Centre for Growth and Development, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, 2-6 Park Avenue, Grafton, Auckland, New Zealand

Abstract

Fetal growth is dependent on the ability of the mother to provide adequate nutritional support to the placenta and the fetus and the ability of the fetus to manage the maternal resources appropriately. The long-term influence of maternal nutrition and other environmental influences during fetal development on postnatal growth and metabolism in offspring are increasingly recognised. Epidemiological studies have shown that perturbations during fetal life can have persistent health consequences. Both prospective clinical studies and experimental research suggest that the propensity to develop abnormalities of cardiovascular, endocrine and metabolic homeostasis in adulthood is increased when fetal development has been adversely affected. The disease process is not based on genetic defects but on altered genetic expression as a consequence of an adaptation to environmental changes during fetal development. This concept has been termed the "fetal origins hypothesis" and the process which underlies this concept has been termed "fetal programming". One general thesis is that the fetus adapts to adverse environmental conditions *in utero* with permanent readjustments of homeostatic systems to maximise its chances for survival. These adaptations may include resetting of metabolic and endocrine systems that lead to a change of growth trajectory and postnatal health consequences.

The scientific concept of fetal programming provides an intellectual framework that links physiological adaptations that occur during early development to permanent changes during postnatal life. It also offers research tools to investigate the mechanistic basis of altered maternal-placental supply of nutrients to the fetus and its long-term consequences for animal performance and health. While recent research in this area has highlighted some potential mechanisms by which these changes may exert their effects, there is little published information on farm animal performance. The aim of this review therefore is to integrate some of the literature on nutritional influences and other environmental challenges during development as they provide support for various mechanisms of fetal programming of endocrine and metabolic regulatory system. A range of issues will be considered with examples from the literature demonstrating that environmental or nutritive exposures have a crucial role in reproductive performance, fetal growth, postnatal development and long-term health. Scientific progress in these areas and further opportunities for research in farm animals will be discussed.

Keywords: fetal programming, fetal growth, animal performance, partitioning of nutrients, postnatal growth

Introduction

Growth in its broadest sense covers many different physiological processes. At its most basic level, it involves the co-ordinated regulation of cell proliferation, cell death, cell migration and differentiation. Appropriate fetal growth requires integration of the intrinsic genetic programmes with environmental cues and with variations in nutrition. Fetal growth is limited by the mother's capacity to supply nutrients to the placenta and the fetus, and the ability of the fetus to manage the maternal resources. The partitioning of nutrients between mother, placenta and fetus is facilitated by hormones which regulate the distribution, transport and utilisation of available substrate. During pregnancy, changes in maternal appetite, body composition, energy consumption and metabolism facilitate adequate supply of nutrients, oxygen and water to the developing fetus. These adaptive maternal responses to pregnancy are regulated directly or indirectly by endocrine mechanisms. Hormones such as insulin, GH, insulin-like growth factors (IGFs), thyroid hormones, leptin and many others of maternal, placental or fetal origin regulate the partitioning of nutrients between the maternal, placental and fetal compartments. Placental hormones such as progesterone, estrogens and placental lactogen are largely secreted into the maternal circulation to modify maternal metabolism. They have also been implicated in the regulation of placental and fetal metabolism.

Optimal growth of the fetus is dependent on the supply of substrates such as glucose, amino acids and lactate from the mother. When we consider fetal substrate supply, it is useful to keep in mind that the fetus is at the endpoint of a quite complex supply line (Harding, 2001). This supply line links maternal nutrition at one end with fetal tissue uptake at the other. Even if current maternal nutrition is optimal, there are a lot of places along this supply line where substrate supply can be interrupted. Impaired maternal nutrient intake is associated with a reduced fetal growth rate, although this can be restored in the short term by maternal re-feeding as long as the undernutrition has not been too prolonged or severe (Harding, 2001). Since maternal nutrients are transferred to the fetus via the placenta, fetal growth is also limited by changes in uterine blood flow as observed with hypertension or acute stress and by other environmental factors that impair placental development or function. In some situations the placenta uses over half of its nutrient resources to support its own metabolic requirements. Intrauterine growth restriction is often associated with large placental size and may therefore arise due to the placenta increasing its nutrient requirements at the expense of resources usually available to the fetus (Owens *et al*., 1989a). Placental function, metabolism and transport of nutrients can be influenced by countless environmental factors, and the consequences of interrupted blood flow to the fetus are a topic of intense clinical and biomedical study (Bennet *et al*., 2001).

The fetus receives the majority of its glucose from the maternal circulation by facilitated diffusion across the placenta, driven by a maternal - fetal concentration gradient. Transfer of amino acids across the placenta occurs by active transport, resulting in high levels of amino acids in the fetal circulation. The primary importance of glucose in late gestation fetal growth is seen in its downstream effects on the fate of other substrates such as amino acids. In well-growing fetuses, glucose levels are high and amino acids are incorporated into protein. However, maternal undernutrition lowers available glucose such that circulating amino acids are then metabolised as an alternative energy source for the feto-placental unit, increasing fetal plasma urea concentrations. With severe undernutrition,

the fetus can undergo tissue wasting and the amino acids which are released are used to maintain placental metabolism (Owens *et al*., 1989b). In general, the more severe and the closer a disruption of the supply line to the fetus, the more immediate and the more dramatic is the fetal response. In response to different insults, the fetus requires an effective repertoire of responses to assure survival. When we consider the nature of fetal survival mechanisms, it will become plausible that changes in fetal substrate supply can have life-long consequences. This phenomenon which we describe as fetal programming is one of the central themes of this paper.

Maternal and fetal responses to reduced maternal nutrition

Maternal fasting or severe undernutrition during late gestation causes fetal growth retardation in the rat and other mammals. Immature and small-for-gestational-age human newborns as well as off-season sheep fetuses that are exposed to suboptimal nutritional conditions show marked growth retardation at birth. It has been shown by Mellor and Matheson (1979) more than 20 years ago that the fetus can slow its growth rate when maternal nutrition is reduced. If the reduction of maternal nutrition is of a reasonably short period, fetal growth can catch up when maternal re-feeding is initiated. If undernutrition extends over long periods of time, the fetus will be growth retarded at birth. However, it is now increasingly recognised that birth weight alone is not a reliable indicator of whether a major disruption in substrate supply has occurred during fetal life (Harding, 2001).

It is now recognised that the timing of alterations in substrate supply during fetal development can lead to quite different birth phenotypes. We have investigated endocrine and metabolic responses of ewes and their fetuses in response to undernutrtion during late gestation. Singleton-bearing ewes were fed either *ad libitum* or 25% of the recommended energy and protein requirements from day 100 until 124 of gestation (Bauer *et al*., 1995). Long-term maternal feed restriction during late gestation resulted in reduced fetal growth, alterations in organ weight and significantly lower body weight close to term. The endocrine and metabolic profiles of both mother and fetus also changed substantially during maternal undernutrition. Maternal and fetal glucose concentrations fell in response to maternal feed restriction. The free fatty acids (FFA) levels were increased in underfed dams whereas the considerably lower fetal FFA concentrations remained unchanged. Interestingly, the effects on the maternal and fetal somatotropic axes were strikingly similar. Both maternal and fetal GH concentrations were elevated with maternal undernutrition, whereas IGF-I concentrations were decreased. This was one of the first studies that clearly showed that the fetal endocrine system in late gestation is nutritionally regulated. This study also suggested that the fetal endocrine system (through changes in circulating GH and IGF-I) may be actively involved in regulating distribution and optimal use of substrates during fetal development.

A different fetal phenotype is observed when the level of maternal nutrition is reduced during early gestation. Bispham *et al*. (2003) investigated the effects of reduced nutrition in pregnant ewes during the period of most rapid placental growth, from approximately day 28 to day 80 of pregnancy. Maternal body weights diverged clearly when pregnant ewes were fed at approximately 50 % of calculated requirements. With re-feeding at day 80 of gestation, ewes' bodyweights increased again to meet the *ad libitum* fed group. Fetuses that were undernourished during the early part of pregnancy had normal body weights close to term; they showed catch-up growth.

However, this catch-up in body weight was reflected in increased adipose tissue weight; the body composition of the fetuses changed as a consequence of maternal undernutrition during early fetal development. As discussed below, the change in maternal endocrine and metabolic milieu during the course of the study illustrates the dynamic nature of the regulatory system that shapes major change in birth phenotype. Interestingly, maternal plasma glucose concentrations were not changed by feed restriction during early gestation while other metabolites (e.g. FFA) did change such that the placenta and consequently the fetus experienced a different metabolic milieu. The small reduction in plasma cortisol in underfed ewes of this study may indicate an absence of a chronic stress response. However, a major reduction in plasma leptin concentrations in undernourished ewes may signal significant changes in metabolic regulation. Leptin is an important hormone for the regulation of intermediary metabolism and plays a central role in appetite regulation. In most mammals, plasma leptin concentrations demonstrate a characteristic rise during mid-pregnancy and a decline towards the end of pregnancy. While leptin's exact role during pregnancy is currently debated, it is tempting to speculate that it may play a role in the communication of metabolic status from the mother to the placenta and the fetus. If the naturally occurring rise in maternal leptin during mid-pregnancy is a signal from the mother to the placenta and the fetus, the fetus of an underfed mother would not receive this signal. This concept is important for our understanding of the role of leptin (and other endocrine signals) during pregnancy when we discuss the postnatal metabolic phenotype that arises when fetal substrate supply is impaired during critical periods of development.

Influence of early life nutrition on postnatal growth and metabolism

Since the developing fetus is totally dependent on the mother for nutritional support it seems plausible that inappropriate nutrition in utero will result in serious consequences for the offspring. It has been known for many years that alterations in nutrition of farm animals before and during pregnancy can significantly influence pregnancy outcome (Everitt, 1968; Downing and Scaramuzzi, 1991). Similarly, the long-term benefits of adequate nutrition before and during lactation on offspring survival and performance are well established (Schinckel and Short 1961; Nottle *et al*. 1998). As discussed below, the long-term effect on offspring performance in farm animals are not known and warrant further study.

Epidemiological studies during the 1980s of adverse health outcomes in humans in which size at birth was related to the occurrence of metabolic or cardiovascular disorders at an adult age (Godfrey and Barker, 2000) have lead to increasing interest in the long-term consequences of nutrition during pregnancy. Many later studies showed that small size or thinness at birth are associated with an increased propensity to adverse health outcomes in adulthood including obesity, type 2 diabetes, abnormal lipid and carbohydrate metabolism, coronary heart disease and elevated blood pressure (Barker, 2000). After initial controversy when these relationships were first suggested, both prospective clinical studies and experimental approaches have shown that the propensity to develop abnormalities of cardiovascular, endocrine and metabolic homeostasis in adulthood is increased when fetal development has been adversely affected (Godfrey and Barker, 2000). This concept has been termed the "fetal origins of adult disease" (FOAD) hypothesis, and the process which remains poorly understood, has been termed "fetal programming". The disease process is not based on genetic defects but on altered genetic expression, which occurs

as a result of fetal adaptations to adverse intrauterine influences. The FOAD hypothesis is now a widely accepted research concept with a growing effort to unravel its biological and mechanistic basis. It is increasingly clear that change in maternal nutrition and consequently altered materno-placental supply of nutrients alters fetal metabolism and endocrine status, with major postnatal health consequences compatible with the FOAD hypothesis. The mechanisms underlying the relationship between prenatal influences and postnatal outcome are relatively unknown and remain speculative. One general thesis is that in response to an adverse intrauterine stimulus, the fetus adapts its development to maximise its immediate chances for survival (Gluckman, 2001). These adaptations may include resetting of metabolic homeostasis and endocrine systems and down-regulation of growth, commonly reflected in altered birth phenotype. This prenatal plasticity of the fetus may allow environmental factors to alter the physiological function of the fetus in preparation for sub-optimal environmental conditions after birth. It is thought that whilst these changes in fetal physiology may be beneficial for short-term survival *in utero*, they may be maladaptive in postnatal life, contributing to poor health outcomes when offspring are exposed to catch up growth, diet-induced obesity and other lifestyle factors.

Animal models of nutritional programming

Animal models are essential in the search for the mechanistic links between prenatal influences and pathophysiological complications in later life. Reduced substrate supply to the fetus has been highlighted as a primary factor involved in the early life origins of adult disease. In the laboratory setting, reduced substrate supply to the fetus can effectively and reliably be achieved through maternal dietary manipulations during pregnancy. Rodent models investigating the mechanistic links between maternal undernutrition and adult disease commonly use either global undernutrition or isocaloric low protein nutrition. Maternal low protein nutrition during pregnancy and lactation is one of the most extensively used tools of nutritional programming. This model involves *ad libitum* feeding of pregnant rats on a low protein diet containing 5-8% protein, but equivalent in energy in comparison to a control diet containing 18-20% protein (Snoeck *et al*., 1990; Langley and Jackson, 1994). Offspring from protein-restricted mothers are approximately 15-20% lighter at birth. Continuation of this low-protein diet during lactation enhances this weight difference. Offspring develop elevated blood pressure at an early age. However, this finding has not been consistent and is related to differences in the composition of the low protein diet (Langley-Evans, 2000). Carbohydrate metabolism in offspring is also altered by a low protein diet during pregnancy in rats. Fasting plasma insulin and glucose levels are lower and are associated with improved insulin sensitivity in early adulthood, but glucose intolerance develops at an older age.

Global undernutrition at various times during pregnancy is another widely-used approach to induce nutritional programming in rats. Several approaches have been developed with different levels of undernutrition during different periods of pregnancy. A mild nutritional restriction to 70% of normal intake in the first 18 days of pregnancy in the rat results in offspring with significant growth retardation at birth that catch up to controls during the early postnatal period (Ozaki *et al*., 2001). Restricted offspring exhibit elevated blood pressure in adult life. Another approach using nutritional restriction to 50% of standard ad libitum intake in the second half of

gestation had no effect on blood pressure, but showed that 21-day-old rat fetuses had significantly decreased pancreatic insulin content (Holemans *et al*., 1999).

We have used maternal undernutrition throughout pregnancy in the rat to investigate the mechanistic basis of the FOAD hypothesis (Woodall *et al*., 1996a; Woodall *et al*., 1996b). On day one of pregnancy animals are randomly assigned to a standard rat chow *ad libitum* throughout pregnancy (*ad libitum* (AD) group) or 30% of the AD group intake of the standard diet throughout gestation (undernourished, UN group). The number of pups borne per litter from UN and AD mothers is identical in this experimental approach; it is not affected by maternal undernutrition. After birth, litter size is adjusted to 8 pups per litter. The UN offspring are cross-fostered within 24 hours of birth onto AD dams to assure adequate and standardised nutrition from birth until weaning. At birth offspring of UN mothers had fetal and placental weights that were 25–30% lower than offspring of AD mothers. Our earlier studies showed a lack of catch-up growth during the early postnatal period despite optimal neonatal nutrition, accompanied by a transient reduction in circulating IGF-I and hepatic IGF-I mRNA expression. Consistent with this observation, we also showed that UN offspring had a reduced responsiveness to GH treatment during the neonatal period, possibly reflecting delayed maturation of the somatotrophic axis, which was fully restored before puberty. In addition, UN offspring developed elevated blood pressure in adult life (Woodall *et al*., 1998). Hypertension and insulin resistance have been induced in rat offspring by maternal undernutrition (Woodall *et al*., 1996a; Vickers *et al*., 2000), a low protein diet (Langley–Evans *et al*., 1996), maternal uterine artery ligation (Rajakumar *et al*., 1998) or maternal dexamethasone (DEX) (Nyirenda *et al*., 1998) treatment. Postnatal obesity has been observed in adult rats after maternal undernutrition or protein restriction during pregnancy. As discussed below, we showed in the rat that feeding offspring of undernourished mothers a hypercaloric diet amplified prenatal influences on hypertension, hyperinsulinemia and obesity (Vickers *et al*., 2000).

There are also increasing experimental data in other species. In guinea pigs intrauterine growth restriction caused by uterine artery ligation or maternal undernutrition results in reduced glucose tolerance, increased sensitivity to cholesterol loading and elevated blood pressure (Kind *et al*., 1999). DEX treatment of pregnant ewes in early gestation results in elevated blood pressure (Dodic *et al*., 1999) and altered regulation of lipolysis (Gatford *et al*., 2000) in the adult offspring. In addition, feed restriction in pregnant sheep leads to alterations in fetal blood pressure, hepatic and renal gluconeogenic enzyme activity and alterations in metabolic and endocrine function (Oliver *et al*., 2001). Undernutrition for 10 days in late gestation ewes alters postnatal HPA axis function of their lambs, but not glucose metabolism. However, when the period of prenatal undernutrition is extended to 20 days, glucose metabolism is altered in adult offspring, but not HPA axis function (Oliver *et al*., 2002; Bloomfield *et al*., 2003).

Interactions between prenatal and postnatal nutrition

Increasing evidence suggests that diet-induced obesity during postnatal life amplifies prenatal influences on susceptibility to disease. In historically undernourished, recently urbanised populations such as India, where children of low birth weight are exposed to a high-fat Western diet, the incidence of obesity and type 2 diabetes is reaching epidemic proportions (Yajnik,

2000). Such observations have been explained by the "thrifty-phenotype" hypothesis proposed by Hales and Barker (1992) and may illustrate the long-term disadvantage of postnatal "catch-up" growth. Although there is considerable debate whether catch-up growth in early postnatal life is beneficial or not, many studies suggested that postnatal "catch-up" growth in children is associated with adverse outcomes in later life (Eriksson *et al*., 1999). Our collaborative studies with the Southampton group have suggested that in cohorts of children in India, small size at birth and rapid postnatal growth are associated with higher blood pressure (Fall *et al*., 1995).

Epidemiological studies have shown that the greatest insulin resistance is observed in people of low birth weight who develop obesity as adults (Phillips, 1998). In rats, the combination of prenatal undernutrition with retarded fetal growth, and good postnatal nutrition with accelerated growth, leads to a striking reduction in life span (Jennings *et al*., 1999). The well-established notion that diets high in saturated fats play a key role in the development of insulin resistance and obesity has recently been extended to the frequency of food intake. Zammit *et al*., (2001) suggested that the pathogenesis of insulin resistance may be related to a pattern of frequent "snacking" which results in a continuous post-prandial state for most of the day. This prevents the establishment of low basal inter-prandial insulin levels even in normal individuals. Prolonged exposure of the liver to high basal insulin, through its stimulatory effect on hepatic triglycerides may contribute to the initial induction of insulin resistance (Zammit *et al*., 2001).

In our studies, we introduced offspring of undernourished rats to a hypercaloric (high fat / high protein) diet after weaning to investigate whether enhanced nutritional supply would facilitate postnatal catch-up growth (Vickers *et al*., 2000). This led to development of obesity during adult life. UN offspring also developed hypertension, hyperinsulinemia, hyperleptinemia and hyperphagia independent of postnatal diet. Postnatal hypercaloric nutrition amplified the existing cardiovascular, metabolic and endocrine abnormalities of UN offspring. Interestingly, hyperphagia was established before puberty independent of caloric content of the diet and increased with advancing age. The increased plasma insulin and leptin concentrations were paralleled by altered pancreatic histology (Vickers *et al*., 2001a; Vickers *et al*., 2001b). The hyperleptinemia and hyperinsulinemia in combination with the hyperphagia seen in UN offspring have been interpreted as a mechanism, induced by the nutrient-deprived fetal environment, to store large quantities of triglycerides on occasions when food is plentiful, thus representing a competitive advantage in preparation for anticipated periods of nutrient deprivation (Hales and Barker, 1992). However, in conditions in which food is plentiful for long periods, in the laboratory situation *ad libitum* nutrition, this strategy is ultimately detrimental, as the hyperphagia continues and metabolic disorders may develop. We have also reported that maternal undernutrition can induce sedentary behaviour in offspring (Vickers *et al*., 2003). Hyperphagia, sedentary behaviour and concomitant obesity in offspring are amplified by hypercaloric nutrition. In the course of these studies, we noted that the onset of abnormal eating and exercise behaviour occurred prior to puberty, thus preceding the development of obesity. However, our work to date cannot resolve whether the primary defect in this cascade of events is in appetite regulation, intermediary metabolism, or altered leptin or insulin action. We have shown that a therapeutic approach using either IGF-1 or GH treatment can ameliorate obesity, hyperphagia and hypertension induced by maternal undernutrition and postnatal hypercaloric nutrition, but the precise mechanisms underlying these effects are yet to be resolved (Vickers *et al*., 2001a; Vickers *et al*., 2002).

Endocrine and metabolic mechanisms

The precise mechanisms underlying the programming of adult disease by maternal nutrient restriction remain a matter of intense scientific research. Maternal undernutrition or low protein diet during the last week of gestation in the pregnant rat leads to reduction of fetal pancreatic β-cell mass (Petrik *et al*., 1999) and increased apoptosis of immature β-cells (Cherif *et al*., 1998). While insulin-stimulated glucose uptake in adipocytes is increased during early postnatal life due to increased insulin receptor number, there is greater age-dependent loss of glucose tolerance and later insulin resistance. The reduced insulin action is associated with reduced phosphatidylinositol 3-kinase (PI3K), and protein kinase B (PKB) activation and altered fatty acid metabolism (Ozanne *et al*., 1998; Ozanne *et al*., 2001).

Another important target of prenatal events is the liver, where glucocorticoids regulate several metabolic processes, including hepatic enzymes regulating carbohydrate and fat metabolism. Rats exposed to DEX in the last trimester of pregnancy show increased phosphoenolpyruvate carboxykinase (PEPCK) gene transcription and increased activity of this rate-limiting enzyme of gluconeogenesis in the liver (Nyirenda *et al*., 1998). These animals have adult hyperglycemia and increased hepatic glucocorticoid receptor expression. Similarly, structural changes in the liver and altered expression patterns of gluconeogenic enzymes and glucose handling have been reported after a maternal low protein diet (Burns *et al*., 1997).

Neuroendocrine regulatory systems are also vulnerable to disturbances in early life which can lead to permanent structural changes, including reduced cerebral vascularity (Bennis-Taleb *et al*., 1999) and dysfunction of central nervous system regulation. Maternal low protein nutrition results in structural changes in the mediobasal hypothalamic nuclei in weanling offspring and fewer neurons immunopositive for neuropeptide Y in the arcuate hypothalamic nucleus (Plagemann *et al*., 2000). These neuroendocrine changes are accompanied by the development of obesity and diabetogenic disturbances later in life. The HPA axis is particularly susceptible to prenatal influences. For example, neonatal disturbance of mother-pup interactions permanently alters plasma adrenocorticotropic hormone (ACTH) and corticosterone concentrations and glucocorticoid receptor (GR) levels in hippocampus, paraventricular nucleus (PVN) and pituitary (Meaney, 2001). Similar GR abnormalities have been described following either nutritional manipulation or glucocorticoid administration to the mother. Maternal glucocorticoids alter the utilisation of promotor regions on the GR gene in offspring (McCormick *et al*., 2000). This is strong evidence that fetal programming can cause permanent alterations in gene expression and could explain the increase in basal plasma corticosterone levels in adulthood that may contribute directly to hypertension and hyperglycemia.

As discussed above, our own research investigating interactions between pre- and postnatal nutrition and other studies that have examined diet-induced obesity point to a link between peripheral leptin resistance and insulin resistance in the development of obesity. The physiological role of hyperleptinemia associated with caloric excess has been proposed to relate to the protection of non-adipocytes from lipid oversupply that would lead to steatosis and lipotoxicity (Unger and Orci, 2001). Elevated leptin production as a result of short-term caloric excess prevents the up-regulation of lipogenesis and increases fatty acid oxidation, thus reducing lipid supply to

peripheral tissue during caloric excess. In diet-induced obesity, peripheral leptin function is at first normal. However, prolonged caloric excess results in dysregulation of post-receptor leptin signalling. This causes accumulation of triglycerides and lipid metabolites, providing fatty acid substrate for the damaging effects of non-oxidative metabolism leading to functional impairment of non-adipose tissue and a progression to type 2 diabetes and cardiovascular disease (Unger, 2001).

Leptin acts at the level of the hypothalamus to regulate appetite and energy homeostasis (Ahima and Flier, 2000). The long-form or signalling form of the leptin receptor (OB-Rb) is expressed in high levels in several cell groups of the hypothalamus and in various tissues throughout the body. Under normal physiological conditions, increased leptin signalling in the medial hypothalamus is associated with reduced neuropeptide Y (NPY) and agouti-related (AgRP) protein production (Hahn *et al*., 1998) but increased cocaine- and amphetamine- regulated transcript (CART) and pro-opiomelanocortin (POMC) production (Schwartz *et al*., 1997). These leptin-induced changes in neuropeptides lead to decreased food intake and increased energy expenditure. In obese individuals, elevated plasma leptin is proposed to uncouple leptin action on its receptors in the hypothalamus, thereby disrupting signal transduction pathways that exert effects on satiety and energy expenditure (Ahima and Flier, 2000). Direct leptin signalling in peripheral tissues has also been demonstrated. For example, increased leptin signalling in muscle tissue has been shown to blunt lipogenesis and stimulate lipid oxidation. There is also growing evidence for a feedback system between leptin and insulin which links the brain and the endocrine pancreas with other peripheral insulin and leptin sensitive tissues in the control of feeding behaviour, metabolic regulation and body energy balance (Kieffer and Habener, 2000).

The world-wide interest in obesity and metabolic regulation coupled with the development of new research approaches has stimulated fetal programming-related research in a growing number of laboratories. Further research is needed to explore potential benefits for agricultural production strategies that may enhance farm animal performance.

Fetal programming - opportunities for research in farm animals

The benefits of appropriate maternal nutrition (Everitt, 1968) on litter size, birth weight and on subsequent growth during rearing by the dam (Taplin and Everitt, 1964) have been accepted for many years. However, only few experiments have systematically investigated the long-term effects of reduced substrate supply to the fetus on the effects of postnatal performance. Recent studies by Greenwood *et al.* (1998) investigated the interactions of impaired prenatal development and postnatal nutrition on growth characteristic in weanling lambs. Low birth weight lambs from litters of multiple lambs were individually reared on a high quality liquid diet to grow rapidly. The main findings of this study were that reduced substrate supply during fetal development had major carry-over effects on postnatal growth. Increased body fat composition and reduced energy efficiency were some of the most important disadvantages of low birth weight lambs. In agreement with results from studies in rodents, increasing nutrition to these low birth weight lambs during the pre-weaning period resulted in enhanced growth rates but it also increased fatness. The authors concluded that implementation of such strategies would need to be balanced against additional costs.

An effective animal husbandry approach to increase the birth weight and post-weaning survival of twin lambs is mid-pregnancy shearing (Symonds *et al.*, 1986; Morris and McCutcheon, 1997). Shearing ewes during mid-pregnancy has been shown to consistently increase lamb birth weight in a variety of farming systems. Mid-pregnancy shearing is associated with a marked increase in the birth weight of twin-born lambs, and in some studies the birth weight of singletons was also increased. The mechanisms underlying the enhanced fetal growth after shearing of the pregnant ewe have been attributed to a range of factors including cold exposure, increase in voluntary feed intake by the ewe and increased nutrient partitioning to the fetus. A recent study by Revell *et al.* (2000) suggests that the increase in lamb birth weight after mid-pregnancy shearing may be associated with reduced maternal glucose utilization and an increased non-insulin dependent uptake of glucose by the placental-fetal unit. This experimental approach may be useful for further work on the regulation of placental glucose uptake and the partitioning of nutrients between mother, placenta and fetus. Future studies should also address the long-term effects in offspring on growth performance, long-term health and other economic traits.

Another example of increasing reproductive efficiency of pastured-based sheep production systems is the use of grazing *Lotus corniculatus* before ovulation and during the first weeks of pregnancy (Min *et al.*, 2001). *Lotus corniculatus* contains condensed tannins that improve the nutrition of sheep by reducing ruminal degradation of plant protein and increasing crude protein flow to the intestine. A significant increase in ovulation rate, lambs born per ewe and increased weaning performance has been reported for ewes grazing lotus over a period of five to seven weeks. However, long-term follow-up studies need to be performed to assess the potential benefits on offspring performance.

Many studies of nutritional manipulations of the mother before and during pregnancy have shown that fetal growth and new-born survival can indeed be influenced by exogenous factors. However, there are other research tools that can further our understanding of biological principles and potential long-term consequences of changes in partitioning of nutrients between mother, placenta and fetus. Exogenous administration of hormones or manipulations of endogenous endocrine activity by immunization against specific hormones are two examples which have been described recently. Rehfeldt *et al.* (2001) investigated the effects of maternal treatment with porcine somatotropin (pST) during early gestation on embryonic survival, fetal development, and fetal growth. The pST treatment during days 10 – 27 of gestation in pigs lead to an increase in birth weight of those piglets which were in the 25 % lowest weight group within each litter. These are the fetuses that are most affected by maternal constraint and it is tempting to speculate that increased nutrient supply during early gestation may have reset their developmental trajectory. This study also showed an increase in muscle protein in fetuses of pST treated sows. In a similar study carcasses of market-weight progeny from pST-treated sows had a larger longissimus muscle cross-sectional area, and longer carcass sides (Kelley *et al.*, 1995). However, our knowledge about the mechanisms of growth hormone-induced changes in the partitioning of nutrients during gestation and its long-term consequences in offspring is quite limited.

Another line of evidence for the scope of modifying the partitioning of nutrients between mother, placenta and fetus comes from studies employing immunization against endogenous placental lactogen. Many studies in sheep have suggested that ovine placental lactogen (oPL) plays an

important role in regulating fetal growth and development and it has been suggested that oPL may be one of the main endocrine regulators of nutrient partitioning during pregnancy in sheep. Leibovich *et al*., (2000) immunized five-month-old ewe lambs against oPL and showed that all immunized ewes developed anti-oPL immune activity without any effect on the establishment or maintenance of pregnancy. Plasma levels of oPL in immunized ewes increased during gestation to similar levels as observed in control ewes. However, at day 130 of gestation, bioactive oPL was higher in immunized than in control ewes despite the fact that about 50% of the oPL in the circulation of the immunized ewes was bound to anti-oPL antibodies. Immunization against oPL increased birth weight of lambs. Lambs born as singles, twins or triplets to immunized ewes had significantly higher birth weights in comparison to control ewes. Furthermore, immunized ewes produced significantly more milk than control ewes without effects on milk composition. The results of this study showed that alterations in oPL bioavailability may enhance fetal growth and mammogenesis before lambing, as well as milk production during lactation. Further work is required to understand the mode of action and the potential long-term benefits in this experimental paradigm.

Conclusion

A picture is emerging in support of the biological principle that interactions between prenatal and postnatal nutrition can permanently reset the development of the postnatal phenotype. In biomedical research much attention has been devoted to investigating mechanisms that explain the pathogenesis of common metabolic disease states in Western World countries. However, if the biological principle of the fetal origins hypothesis is valid across a range of mammalian species, it makes sense to explore whether this research strategy can be employed for the benefit of farming systems. Research in this area provides a scientific framework that links physiological adaptations during early development to permanent changes in postnatal life. It offers research tools to investigate the mechanistic basis of altered maternal-placental supply of nutrients to the fetus and its long-term consequences for animal performance and health. The scientific goals of such research should recognize the needs of sustainable agricultural systems, environmental and economic benefits, animal welfare, ethical considerations and consumer acceptance. Recent reviews of the literature describe a range of animal production strategies that are well situated to incorporate fetal programming research in farm animals into a "clean, green and ethical" concept (Martin *et al*., 2004).

Acknowledgements

The author gratefully acknowledges support from the Health Research Council of New Zealand and the National Research Centre for Growth and Development.

References

Ahima, R.S. & J.S. Flier (2000). Leptin. Annual Review of Physiology, 6, 413-437.

Barker, D.J. (2000). In utero programming of cardiovascular disease. Theriogenology, 53, 555-574.

Bauer, M., B.H. Breier, J.E. Harding, J.D. Veldhuis & P.D. Gluckman (1995). The fetal somatotropic axis during long-term undernutrition in sheep: evidence for nutritional regulation in utero. Endocrinology, 136, 1250-1257.

Bennet, L., M.H. Oliver, A.J. Gunn, M. Hennies & B.H. Breier (2001). Differential regulation of insulin-like growth factor (IGF) – 1 and IGF-2 and their binding proteins following asphyxia in the preterm fetal sheep. Journal of Physiology, 531, 835-841.

Bennis-Taleb, N., C. Remacle, J.J. Hoet & B. Reusens (1999). A low-protein isocaloric diet during gestation affects brain development and alters permanently cerebral cortex blood vessels in rat offspring. Journal of Nutrition, 129, 1613-19.

Bispham, J., G.S. Gopalakrishnan, J. Dandrea, V. Wilson, H. Budge, D.H. Keisler, P.F. Broughton, T. Stephenson & M.E. Symonds (2003). Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. Endocrinology, 144, 3575-3585.

Bloomfield, F.H., M.H. Oliver, C.D. Giannoulias, P.D. Gluckman, J.E. Harding & J.R. Challis (2003). Brief undernutrition in late-gestation sheep programmes the hypothalamic-pituitary adrenal axis in adult offspring. Endocrinology, 144, 2933-40.

Burns, S.P., M. Desai, R.D. Cohen, C.N. Hales, R.A. Iles & T.C. Going (1997). Gluconeogenesis, glucose handling, and structural changes in livers of the adult offspring of rats partially deprived of protein during pregnancy and lactation. Journal of Clinical Investigation, 100, 1768-1774.

Cherif, H., B. Reusens, M.T. Ahn, J.J. Hoet & C. Remacle (1998). Effects of taurine on the insulin secretion of rat fetal islets from dams fed a low-protein diet. Journal of Endocrinology, 159, 341-348.

Dodic, M., E.M. Wintour, J.A. Whitworth & J.P. Coghlan (1999). Effect of steroid hormones on blood pressure. Clinical & Experimental Pharmacology & Physiology, 26, 550-552.

Downing, J.A. & R.J. Scaramuzzi (1991). Nutrient effects on ovulation rate, ovarian function and the secretion of gonadotrophic and metabolic hormones in sheep. Journal of Reproduction and Fertility, (Suppl) 43, 209-227.

Eriksson, J.G., T. Forsen, J. Tuomilehto, P.D. Winter & D.J. Barker (1999). Catch-up growth in childhood and death from coronary heart disease: longitudinal study. British Medical Journal, 318, 427-431.

Everitt, G.C. (1968). Prenatal development of uniparous animals, with particular reference to the influence of maternal nutrition in sheep. In: G. A. Lodge and G. E. Lamming (Ed.) Growth and Development of Mammals. p 131. Proceedings of the 14th Nottingham Easter School. Butterworths, London.

Fall, C.H., A.N. Pandit, C.M. Law, C.S. Yajnik, P.M. Clark, B. Breier, C. Osmond, A.W. Shiell, P.D. Gluckman & D.J. Barker (1995). Size at birth and plasma insulin-like growth factor-1 concentrations. Archives of Disease in Childhood, 73, 287-293.

Gatford, K.L., E.M. Wintour, M.J. De Blasio, J.A. Owens & M. Dodic (2000). Differential timing for programming of glucose homoeostasis, sensitivity to insulin and blood pressure by in utero exposure to dexamethasone in sheep. Clinical Science, 98, 553-560.

Gluckman, P.D. (2001). Editorial: nutrition, glucocorticoids, birth size, and adult disease. Endocrinology, 142, 1689-1691.

Godfrey, K.M. & D.J.P. Barker (2000). Fetal nutrition and adult disease. American Journal of Clinical Nutrition, 71, 1344s-1352s.

Greenwood, P.L., A.S. Hunt, J.W. Hermanson & A.W. Bell (1998). Effects of birth weight and postnatal nutrition on neonatal sheep: I. Body growth and composition, and some aspects of energetic efficiency. Journal of Animal Science, 76, 2354-2367.

Hahn, T.M., J.F. Breininger, D.G. Baskin & M.W. Schwartz (1998). Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. Nature Neuroscience, 1, 271-272.

Hales, C.N. & D.J. Barker (1992). Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenol type hypothesis. Diabetologia, 35, 595-601.

Holemans, K., R. Gerber, K. Meurrens, F. De Clerck, L. Poston & F.A. Van Assche (1999). Maternal food restriction in the second half of pregnancy affects vascular function but not blood pressure of rat female offspring. British Journal of Nutrition, 81, 73-79.

Harding, J.E. (2001). The nutritional basis of the fetal origins of adult disease. International Journal of Epidemiology, 30, 15-23.

Jennings, B.J., S.E. Ozanne, M.W. Dorling & C.N. Hales (1999). Early growth determines longevity in male rats and may be related to telomere shortening in the kidney. FEBS Letters, 448, 4-8.

Kelley, R.L., S.B. Jungst, T.E. Spencer, W.F. Owsley, C.H. Rahe & D.R. Mulvaney (1995). Maternal treatment with somatotropin alters embryonic development and early postnatal growth of pigs. Domestic Animal Endocrinology, 12, 83-94.

Kieffer, T.J. & J.F. Habener (2000). The adipoinsular axis: effects of leptin on pancreatic beta-cells. American Journal of Physiology, 278, E1-E14.

Kind, K.L., P.M. Clifton, A.I. Katsman, M. Tsiounis & J.A. Owens (1999). Restricted fetal growth and the response to dietary cholesterol in the guinea pig. American Journal of Physiology, 277, R1675-R1682.

Langley, S.C. & A.A. Jackson (1994). Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. Clinical Science, 86, 217-222.

Langley-Evans, S.C., D.S. Gardner & A.A. Jackson (1996). Association of disproportionate growth of fetal rats in late gestation with raised systolic blood pressure in later life. Journal of Reproduction and Fertility, 106, 307-312.

Langley-Evans, S.C. (2000). Critical differences between two low protein diet protocols in the programming of hypertension in the rat. International Journal of Food Sciences and Nutrition, 51, 11-17.

Leibovich, H., A. Gertler, F.W. Bazer & E. Gootwine (2000). Active immunization of ewes against ovine placental lactogen increases birth weight of lambs and milk production with no adverse effect on conception rate. Animal Reproduction Science, 64, 33-47.

Martin GB, Milton JT, Davidson RH, Banchero Hunzicker GE, Lindsay DR, Blache D. (2004). Natural methods for increasing reproductive efficiency in small ruminants. Animal Reproduction Science, 82-83, 231-245.

McCormick, J.A., V. Lyons, M.D. Jacobson, J. Noble, J. Diorio, M. Nyirenda, S. Weaver, W. Ester, J.L. Yau, M.J. Meaney, J.R. Seckl & K.E. Chapman (2000). 5'-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. Molecular Endocrinology, 14, 506-517.

Meaney, M.J. (2001). Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annual Review of Neuroscience, 24, 1161-1192.

Mellor, D.J. & I.C. Matheson (1979). Daily changes in the curved crown-rump length of individual sheep fetuses during the last 60 days of pregnancy and effects of different levels of maternal nutrition. Quarterly Journal of Experimental Physiology and cognate Medical Sciences, 64, 119-31.

Min, B.R., J.M. Fernandez, T.N. Barry, W.C. McNabb & P.D. Kemp (2001). The effect of condensed tannins in Lotus corniculatus upon reproductive efficiency and wool production in ewes during autum. Animal Feed Science and Techniology, 92, 185-2002.

Morris, S.T. & S.N. McCutcheon (1997). Selective enhancement of growth in twin foetuses by shearing ewes in early gestation. Animal Science, 65, 105-110.

Nottle, M.B., D.O. Kleemann, V.M. Hocking, T.I. Grosser & R.F. Seamark (1998). Development of a nutritional strategy for increasing lamb survival in Merino ewes mated in late spring/early summer. Animal Reproduction Science, 52, 213-219.

Nyirenda, M.J., R.S. Lindsay, C.J. Kenyon, A. Burchell & J.R. Seckl (1998). Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. Journal of Clinical Investigation, 101, 2174-2181.

Oliver, M.H., P. Hawkins, B.H. Breier, P.L. van Zijl, S.A. Sargison & J.E. Harding (2001). Maternal undernutrition during the periconceptual period increases plasma taurine levels and insulin response to glucose but not arginine in the late gestation fetal sheep. Endocrinology, 142, 4576-4579.

Oliver, M.H., B.H. Breier, P.D. Gluckman & J.E. Harding (2002). Birth weight rather than maternal nutrition influences glucose tolerance, blood pressure and IGF-1 levels in sheep. Pediatric Research, 52, 516-524.

Owens, J.A., P.C. Owens & J.S. Robinson (1989a). Experimental growth retardation: metabolic and endocrine aspects. In: Advances in fetal physiology. Ed: PD Gluckman, BM Johnston, and PW Nathanielsz. Perinatology Press, Ithaca. pp. 263-286.

Owens, J.A., J. Falconer & J.S. Robinson (1989b). Glucose metabolism in pregnant sheep when placental growth is restricted. Amican Journal of Physiology, 257, 350-357.

Ozaki, T., H. Nishina, M.A. Hanson & L. Poston (2001). Dietary restriction in pregnant rats causes gender-related hypertension and vascular dysfunction in offspring. Journal of Physiology, 530, 141-152.

Ozanne, S.E., N.D. Martensz, C.J. Petry, C.L. Loizou & C.N. Hales (1998). Maternal low protein diet in rats programmes fatty acid desaturase activities in the offspring. Diabetologia, 41, 1337-1342.

Ozanne, S.E., M.W. Dorling, C.L. Wang & B.T. Nave (2001). Impaired PI 3-kinase activation in adipocytes from early growth-restricted male rats. American Journal of Physiology and Endocrinological Metabolisme, 280, E534-E539.

Petrik, J., B. Reusens, E. Arany, C. Remacle, C. Coelho & D.J. Hill (1999). A low protein di*et al*ters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. Endocrinology, 140, 4861-73.

Phillips, D.I. (1998). Birth weight and the future development of diabetes. A review of the evidence. Diabetes Care, 21 (Suppl) 2, B150-B155.

Plagemann, A., T. Harder, A. Rake, K. Melchior, W. Rohde & G. Dorner (2000). Hypothalamic nuclei are malformed in weanling offspring of low protein malnourished rat dams. Journal of Nutrition, 130, 2582-89.

Rajakumar, P.A, J. He, R.A. Simmons & S.U. Devaskar (1998). Effect of uteroplacental insufficiency upon brain neuropeptide Y and corticotropin-releasing factor gene expression and concentrations. Pediatric Research, 44, 168-174.

Rehfeldt, C., G. Kuhn, G. Nurnberg, E. Kanitz, F. Schneider, M. Beyer, K. Nurnberg & K. Ender (2001). Effects of exogenous somatotropin during early gestation on maternal performance, fetal growth, and compositional traits in pigs. Journal of Animal Science, 79, 1789-1799.

Revell, D.K., S.F. Main, B.H. Breier, Y.H. Cottam, M. Hennies & S.N. McCutcheon (2000). Metabolic responses to mid-pregnancy shearing that are associated with a selective increase in the birth weight of twin lambs. Domestic Animal Endocrinology, 18, 409-22.

Schinckel, P.G. & B.F. Short (1961). The influence of nutritional level during pre-natal and early post-natal life on adult fleece and body characteristics. Australian Journal of Agricultural Research, 12, 176-202.

Schwartz, M.W., R.J. Seeley, S.C. Woods, D.S. Weigle, L.A. Campfield, P. Burn & D.G. Baskin (1997). Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes, 46, 2119-2123.

Snoeck, A., C. Remacle, B. Reusens & J.J. Hoet (1990). Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biology of the Neonate, 57, 107-118.

Symonds, M.E., M.J. Bryant & M.A. Lomax (1986). The effect of shearing on the energy metabolism of the pregnant ewe. British Journal of Nutrition, 56, 635-643.

Taplin, D.E. & G.C. Everitt (1964). The influence of prenatal nutrition on postnatal performance of merino lambs. Proceedings of Australian Society of Animal Production, 5, 72-81.

Thornton, J.E., C.C. Cheung, D.K. Clifton & R.A. Steiner (1997).Regulation of hypothalamic proopiomelanocortin mRNA by leptin in ob/ob mice. Endocrinology, 138, 5063-66.

Unger, R.H. & L. Orci (2001). Lipotoxic diseases of nonadipose tissues in obesity. International Journal of Obesity, 24, Supplement 4, S28-S32.

Unger, R.H. (2001). Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. Diabetes, 44, 863-870.

Vickers, M.H., B.H. Breier, W.S. Cutfield, P.L. Hofman & P.D. Gluckman (2000). Fetal origins of hyperphagia, obesity and hypertension and its postnatal amplification by hypercaloric nutrition. American Journal of Physiology, 279, E83-E87.

Vickers, M.H., S. Reddy, B.A. Ikenasio & B.H. Breier (2001a). Dysregulation of the adipoinsular axis - a mechanism for the pathogenesis of hyperleptinemia and adipogenic diabetes induced by fetal programming. Journal of Endocrinology, 170, 323-332.

Vickers, M.H., B.A. Ikenasio & B.H. Breier (2001b). IGF-1 treatment reduces hyperphagia, obesity, and hypertension in metabolic disorders induced by fetal programming. Endocrinology, 142, 3964-3973.

Vickers, M.H., B.A: Ikenasio & B.H. Breier (2002). Adult growth hormone treatment reduces hypertension and obesity induced by an adverse prenatal environment. Journal of Endocrinology, 175, 615-623.

Vickers, M., B. Breier, D. McCarthy & P.D. Gluckman (2003). Sedentary behavior during postnatal life is determined by the prenatal environment and exacerbated by postnatal hypercaloric nutrition. American Journal of Physiology, 285, R271-R273.

Woodall, S.M., B.M Johnston, B.H. Breier & P.D. Gluckman (1996a). Chronic maternal undernutrition in the rat leads to delayed postnatal growth and elevated blood pressure of offspring. Pediatric Research, 40, 438-443.

Woodall, S.M., B.H. Breier, B.M. Johnston & P.D. Gluckman (1996b). A model of intrauterine growth retardation caused by chronic maternal undernutrition in the rat: effects on the somatotropic axis and postnatal growth. Journal of Endocrinology, 150, 231-242.

Woodall, S.M., N.S. Bassett, P.D. Gluckman & Breier, B.H. (1998). Consequences of maternal undernutrition for fetal and postnatal hepatic insulin-like growth factor-I, growth hormone receptor and growth hormone binding protein gene regulation in the rat. Journal of Molecular Endocrinology, 20, 313-326.

Yajnik, C. (2000). Interactions of perturbations in intrauterine growth and growth during childhood on the risk of adult-onset disease. Proceedings of the Nutrition Society, 59, 257-265.

Zammit, V.A., I.J. Waterman, D. Topping & G. McKay (2001). Insulin stimulation of hepatic triacylglycerol secretion and the etiology of insulin resistance. Journal of Nutrition, 131, 2074-2077.

Mammary cell turnover: relevance to lactation persistency and dry period management

A.V. Capuco[1], E. Annen[2], A.C. Fitzgerald[2], S.E. Ellis[3] and R.J. Collier[2]
[1]USDA-ARS, Bovine Functional Genomics Laboratory, Bldg 200, Beltsville Agricultural Research Center, Beltsville, MD 20705, USA
[2]University of Arizona, Dept. of Animal Sciences, 217 Shantz Building, P.O. Box 210038, Tucson, AZ 85721, USA
[3]Clemson University, Animal and Veterinary Science Department, 124 Poole Agricultural Center, Clemson, SC 29634, USA

Abstract

Milk production is a function of the number of mammary secretory cells and the activity per cell. Declining milk yield with advancing lactation is due to a decline in the number of secretory cells, although a decline in activity per cell occurs during late gestation. By replacing older cells with younger cells, cell turnover is a mechanism whereby persistency of lactation can be enhanced. Management practices such as bovine somatotropin (bST) supplementation, photoperiod manipulation and increased milking frequency may enhance lactation persistency. Mastitis, stressors, concomitant pregnancy, and decreased milking frequency may decrease persistency by increasing apoptosis. Mammary epithelial cells with lightly staining cytoplasm appear to be the progenitor cells responsible for proliferation within the mammary gland. Renewal of these cells and senescent secretory cells prior to the next lactation may be critical events responsible for the impact of dry period length on milk yield in the next lactation. Data indicate that there is extensive cell renewal in conjunction with net proliferation during the dry period. Although the 60-d dry period is the current management standard, continued advances in genetic potential and dairy management indicate that a reassessment is in order. Recent studies have shown that multiparous dairy cows treated with bST can be milked continuously without significant milk loss in the next lactation. Indeed, total milk yields (pre plus postpartum yields) were increased. However, lactating heifers require a dry period of sufficient length for optimal milk yield in the next lactation. Neither bST nor increased milking frequency reversed the negative impact of an inadequate dry period on milk production by dairy heifers. However, these studies have provided additional insights and areas for future investigation.

Keywords: involution, proliferation, apoptosis, progenitor cells, lactation persistency

Introduction

This paper focuses on mammary cell turnover and its relevance to issues of lactation persistency and dry period length. Initial studies demonstrated that the decline in milk yield after peak lactation in goats is due primarily to a decline in the number of secretory cells within the mammary gland, and to a lesser extent to declining secretory capacity per cell (Knight and Peaker, 1984). More recent studies established that the same holds true during a bovine lactation, and that the decline in cell number with advancing lactation is due to apoptotic cell death (Capuco *et al.*,

2001a). Slight alterations in rates of mammary epithelial cell proliferation or apoptosis will have profound effects on the number of mammary epithelial cells (MEC) during lactation and hence the persistency of the lactation. Furthermore, the continued replacement of older cells with younger cells can help maintain the secretory capability of the epithelial cells throughout lactation. At the conclusion of a bovine lactation, the total population of secretory cells is approximately half of that present at the beginning of lactation. This complement of cells must be restored in the final weeks of gestation, which typically falls within the nonlactating interval between consecutive lactations termed the dry period.

Traditional management of dairy cows provides for a 60-day dry period between successive lactations in order to maximize milk production in the next lactation. Certainly, there is ample evidence to indicate that in the absence of a dry period, milk production is reduced in the subsequent lactation (Swanson, 1965; Smith *et al*., 1967; Remond *et al*., 1992). However, recommendations for dry period length were based primarily on retrospective studies and older experiments that utilized cows that were not the high producers prevalent in today's production systems. A more recent experiment characterized changes in cell populations during the dry period and brought into question some of the tenets of dry cow management (Capuco *et al*., 1997). In recent years, Bachman, Collier and Grummer (Bachman, 2002; Gulay *et al*., 2003; Bachman and Schairer, 2003; Grummer and Rastani, 2004; Annen *et al*., 2004a) have emphasized the need to reassess dry cow management and suggested that shorter dry periods are possible, profitable and perhaps preferable. The reader is referred to these excellent reviews for coverage of the many factors involved in evaluating dry period management. In lieu of today's management tools to enhance lactational performance, including use of galactopoietic hormones such as bovine somatotropin (bST), increased milking frequency and photoperiod management, it is important to evaluate the impact of dry period length on lactational performance and interactions of dry period length with various management systems. Ultimately, it is clear that determination of the most appropriate dry period length is an economic decision. As such, the impact of dry period length upon other factors such as feeding management, animal health, animal housing and periparturient care within particular management systems should be weighed in conjunction with effects on milk revenue (yield and composition) and colostrum quality (Bachman and Schairer, 2003; Annen *et al*., 2004a; Grummer and Rastani, 2004).

Concept of cell turnover

Cell number within an organ or tissue depends upon the net sum of rates of cell proliferation and cell death. The mammary gland grows when the rate of proliferation exceeds the rate of cell death, regresses when the rate of cell death exceeds the rate of cell proliferation, and maintains constant cell number when rates of proliferation and death are equal. Whether the mammary gland is growing, regressing or under steady state, the population of cells may undergo varying degrees of cell replacement, or turnover determine by the absolute rates of proliferation and cell death. As depicted in Figure 1, two hypothetical populations of cells may regress at equal rates yet differ with regard to the extent of cell replacement or turnover. Typically the cells that are replaced are older, less vigorous cells. Consequently, increased cell turnover in the mammary gland may aid in maintaining high levels of secretory capacity per cell during lactation or replacement of less productive cells during the dry period. This may be the direct result of generating cells

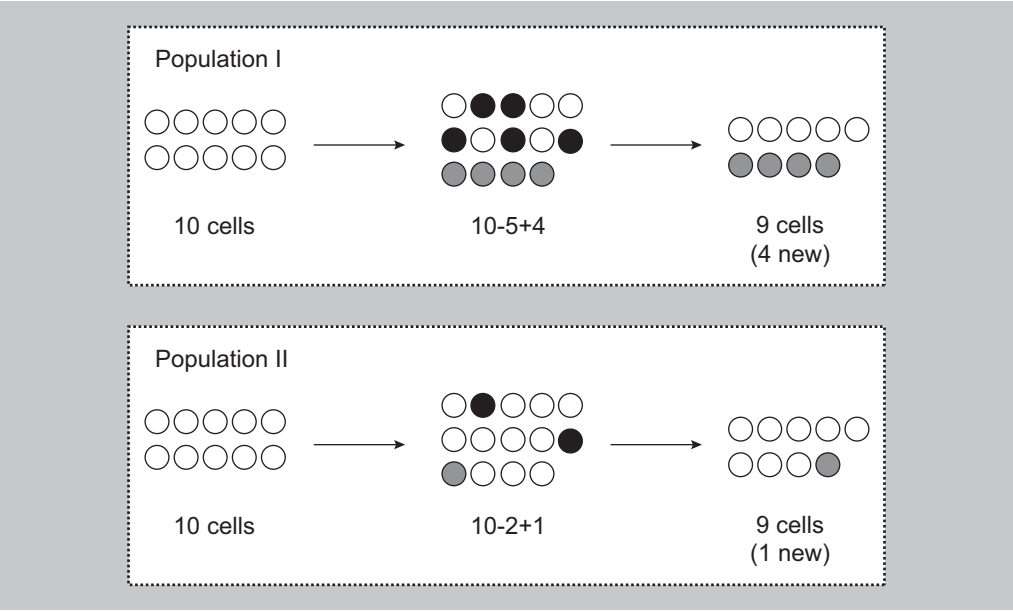

Figure 1. Rates of cell proliferation and cell death determine the net gain or loss of cells and extent of cell renewal within the population. Depicted is a population of cells undergoing net regression. Open circles represent the initial populations of cells, new cells formed by cell proliferation are depicted by grey circles and cells that die during this period are depicted by black circles. In both panels, the net loss is identical because the difference between rates of cell death and proliferation are one cell. However, cell renewal differs markedly between panels. In the upper panel, the population of nine cells contains four new cells. In the lower panel, the population of nine cells contains one new cell. (Source: Capuco et al.*, 2001a)*

with greater secretory capacity or the generation of progenitor cells with enhanced proliferative capacity.

Identification of progenitor cells

There is ample evidence for the existence of mammary stem cells. Convincingly, it has been demonstrated that an entire mammary gland could be regenerated with the progeny of a single cell following transplantation into cleared mammary fat pads (Kordon and Smith, 1998). Other transplantation experiments have shown that tissue fragments isolated from any portion of the mammary gland, during growth or lactational stages, are capable of regenerating a complete mammary ductal and alveolar network (DeOme *et al.*, 1959; Hogg *et al.*, 1983; Smith and Medina, 1988). Additional evidence for the existence of mammary stem cells may be derived from observations that entire mammary lobules are often comprised of cells showing identical X-inactivation patterns and from cancer studies where mammary tumors comprised of a variety of cell types are frequently found to be of clonal origin (Tsai *et al.*, 1996). In addition to pluripotent adult stem cells, evidence exists for mammary progenitor cells that possess a more limited

differentiation capacity, that is they give rise to a more restricted number of cell types and they are not immortal (Chepko and Smith, 1997; Chepko and Smith, 1999).

Histological analyses have indicated that cell populations with lightly staining cytoplasm are present during all stages of mammary development and differentiation in mice and rats, and these may serve as mammary stem cells capable of regenerating all cell types within the mammary gland or as progenitor cells (Chepko and Smith, 1997). Such, "pale cells" have been described in mammary tissue from all species so far examined, including humans (Ferguson, 1985), mice (Smith and Medina, 1988), rats (Chepko and Smith, 1997), goats (Li *et al*., 1999b), and cattle (Ellis *et al*., 2000; Ellis and Capuco, 2002). In bovine mammary gland these lightly staining cells can be distinguished during all phases of development including prepubertal, primigravid, lactation and dry periods. Examples of lightly staining epithelial cells in the bovine mammary gland are depicted in Figure 2.

To test the hypothesis that MEC exhibiting light cytoplasmic staining represented stem cells or progenitor cells, we evaluated the proliferative capacity of these cells in mammary glands of prepubertal heifers (Ellis and Capuco, 2002). Prepubertal heifers were used because of the active proliferative state of the mammary gland during this stage of mammary development (Sinha and Tucker, 1969). We observed light, dark, and intermediate staining cells in histological sections (Figure 2A, B). Light cells comprised 10% of the total parenchymal cell population, but

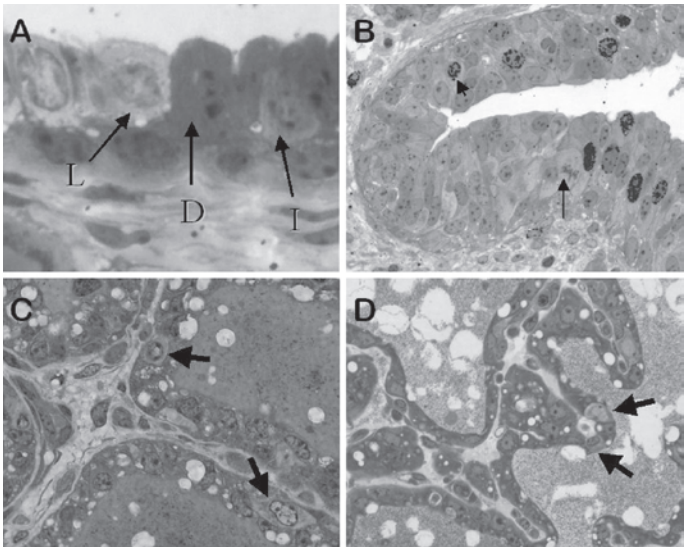

Figure 2. Lightly staining cells in bovine mammary epithelium. Panel A: Light (L), Intermediate (I) and dark (D) cells of a 2 month-old calf. Panel B: Mammary epithelium of a 2 month-old calf, with numerous lightly staining epithelial cells. A mitotic cell is indicated (long arrow) and one of 10 cells labeled with BrdU is indicated (short arrow). Panel C: Mammary epithelium of a dry cow, approximately one week prepartum, with lightly staining cells indicated by arrows. Panel D: Lactating murine mammary epithelium with lightly staining cell indicated by arrows. (Source: Capuco et al*., 2003).*

accounted for the majority of epithelial cell proliferation, assessed by in vivo bromodeoxyuridine (BrdU; a thymidine analog) incorporation and subsequent immunohistochemical visualization. Light and intermediate cells together accounted for more than 90% of the proliferating cells. The proportion of light cells was relatively constant across the stages of development evaluated (2, 5, and 8 months). However, the proportion of intermediate staining cells correlated with the tissue's proliferative rate (Figure 3). These data strongly support the hypothesis that lightly staining MEC function as the primary proliferative population and provide solid justification for further studies into the light staining cell phenotype. We hypothesize that the lightly staining cells provide the basal population of mammary epithelial progenitor or stem cells and that the intermediately staining cells represent a daughter population that serves to amplify the proliferative potential to meet growth demands and may have a more restricted developmental potential. The dark cells represent more differentiated MEC.

Future studies will employ a variety of approaches to identify differences in gene expression that are related to the mammary epithelial cell's staining characteristics. Development of markers for these cells would facilitate kinetic analyses of the relationship between the different staining phenotypes and permit identification of factors that regulate the proliferation of these cells. Such knowledge should provide a key for regulating mammary development and persistency of lactation as these cells represent the proliferative population capable of maintaining cell number during lactation and increasing cell number and turnover during the dry period, as described subsequently.

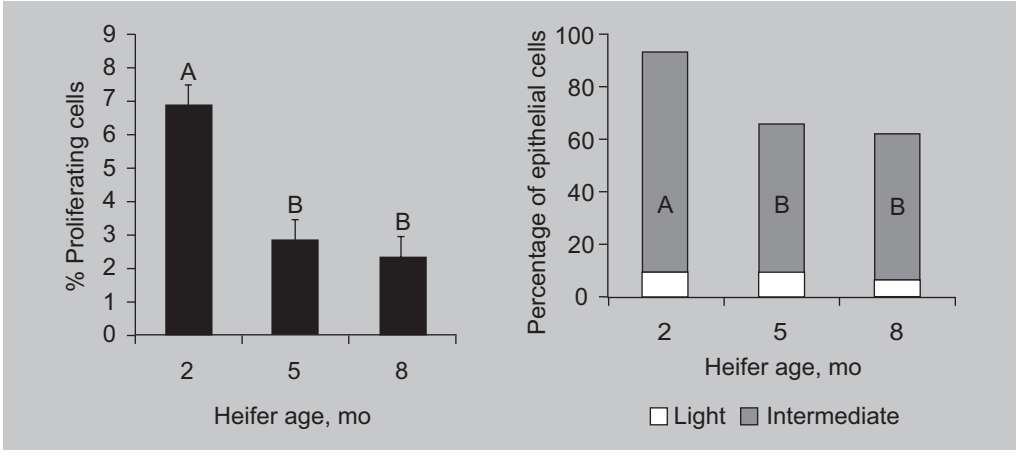

Figure 3. Proliferation of MEC of Holstein heifers (left panel) and percent light and intermediately staining epithelium. The percentage of proliferating cells represents those cells that were labeled with in vivo injection of BrdU plus those cells that were mitotic. The percentage of light and intermediate staining cells is depicted in the right panel. Bars without common superscripts differ ($P < 0.05$) (Source: adapted from Ellis and Capuco, 2002).

Population dynamics during lactation

Knight and Peaker estimated mammary cell number and secretory activity during lactation of goats (Knight and Peaker, 1984). This was accomplished by using multiple biopsies to evaluate changes in nucleic acid concentrations and enzymatic activity. When coupled with measures of udder volume, the data were extrapolated to whole udder measures. This study demonstrated that increases in milk production during early lactation were first the result of an increase in mammary cell number followed by an increase in secretory activity per cell. After peak lactation, decreased milk yield with advancing lactation was primarily the result of declining cell number. However during late lactation, when goats were concomitantly pregnant, the secretory activity per cell also declined.

Analysis of changes in mammary cell number and secretory activity during a bovine lactation was performed utilizing an approach that also permitted evaluation of mammary cell population kinetics (Capuco *et al*., 2001a). Based upon measures of total udder DNA, increased milk yield during early lactation was attributed to increased secretory activity per cell and the decline in milk yield with advancing lactation due to declining cell number. The secretory activity per cell increased prior to peak lactation but did not change significantly with advancing lactation. These data (Capuco *et al*., 2001a) pertain to nonpregnant multiparous cows. When cows are concomitantly lactating and pregnant, it is likely that a decline in secretory capacity per mammary cell accompanies advanced pregnancy, due to conflicting metabolic demands of gestation and lactation. This is readily apparent during late pregnancy or extended pregnancy, when the number of MEC increases simultaneously with a rapid decline in milk production. Rates of proliferation and apoptosis, estimated by BrdU and terminal dUTP nick end labeling (TUNEL) respectively, are summarized in Figure 4. Rates of mammary cell proliferation were determined by using multiple injections of BrdU to label all cells that synthesize DNA within a 24-h labeling period and then quantifying these cells by immunohistochemistry. The 24-h labeling period provided a measure of average proliferation rate even in the presence of an underlying diurnal pattern of DNA synthesis, such as that reported for rodent mammary glands (Borst and Mahoney, 1980). The in situ labeling of apoptotic cells in histological sections by TUNEL detects cells with fragmented DNA, a hallmark of later stages of apoptosis. Because apoptotic cells appear to persist for about 3 h (Bursch *et al*., 1990), we multiplied the labeling index by eight (24/3) to obtain an estimate for a 24-h apoptotic rate (Capuco *et al*., 2001a).

Measures of proliferation rate and apoptotic rate were consistent with the observed decline in mammary cell number. Although the BrdU-labeling index did not vary significantly during lactation, there was a tendency for reduced rates of cell proliferation during early lactation (14 d). This suggested that the increased mammary cell proliferation evident during gestation and the dry period did not continue during early lactation. The decline in cell number after peak lactation was predicted by the estimates of proliferation and apoptotic rate. Across lactation, 0.3% of mammary cells proliferated per day and the apoptotic index (0.07%) was extrapolated to an apoptotic rate of 0.56% per day. Functions for total cell proliferation and total apoptotic death and net cell number were generated. The predicted decline in cell number closely approximated the decline in mammary DNA observed (Figure 4 and Capuco *et al*., 2001a). The number of cells generated during lactation is predicted to equal the number that are present in the gland at 252 d of lactation

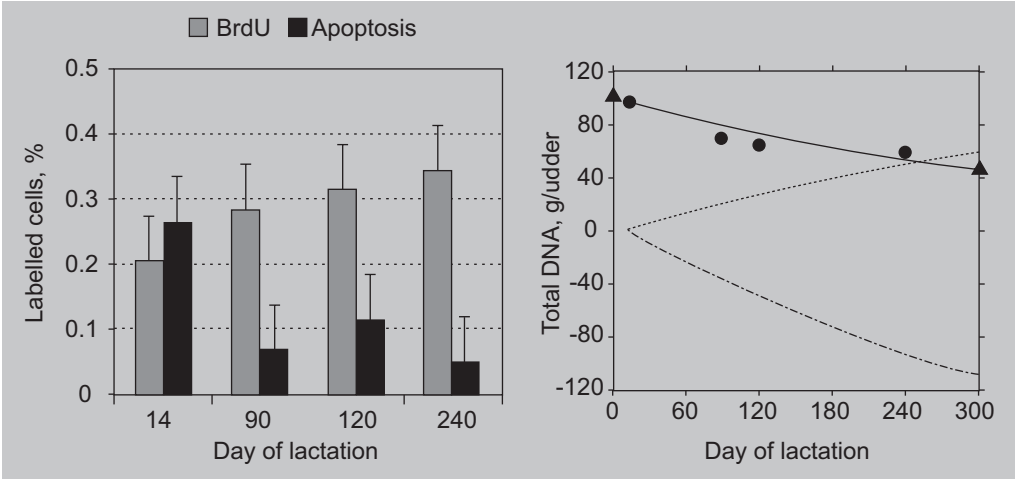

Figure 4. Components of mammary DNA (cell) turnover during lactation. Left Panel: BrdU 24-hour labeling index and TUNEL apoptotic index. The proliferation rate was 0.3% per day (average BrdU 24-h labeling index). The apoptotic index averaged 0.07%; assuming a 3-h duration of apoptosis, the apoptotic rate is 0.56% per day. Right Panel: The accumulated synthesis of new DNA (formation of new cells) is indicated by the dotted line. The accumulated loss of DNA by apoptosis is indicated by the dashed-dotted line. Net loss of DNA (difference between synthesis and loss) is shown by the solid line. Experimental data points from two experiments are indicated by the symbols in the right panel (Source: adapted from Capuco et al.*, 2001a).*

(intersection of cell proliferation and death curves). If none of the cells that died by apoptosis were those that proliferated during lactation then 100% of the mammary cells remaining after 252 d of lactation were formed during lactation. Despite seemingly low proliferation and apoptotic rates during the course of a bovine lactation, extensive cell turnover was observed. This is consistent with recent studies of neural regeneration, for which the cell proliferation rate is exceedingly low but regeneration occurs with appropriate therapy and an extended timeframe. This provided the first quantitative demonstration that apoptotic death could account for the decline in mammary cell number during lactation and this view was further supported by the demonstration that cell loss in milk accounted for less than 1.6% of the decline in cell number (Capuco *et al.*, 2001a).

The extent of mammary cell turnover during a bovine lactation differs from that occurring during lactation in rats. In contrast to the cell turnover during a bovine lactation, approximately 75% of mammary cells are maintained throughout an entire lactation in rats (Pitkow *et al.*, 1972). This lack of extensive cell turnover may be due to the shorter length of lactation in rodents compared with cows.

A number of factors can influence persistency of lactation, are outlined in Figure 5 and more fully described in a recent review (Capuco *et al.*, 2003). Among the factors with the potential to enhance persistency of lactation by increasing cell proliferation or decreasing apoptosis are (1) bST and components of the GH/IGF axis (2) prolactin through potential interactions with IGF survival activity (3) photoperiod, perhaps mediated by prolactin and IGF axis (4) increased

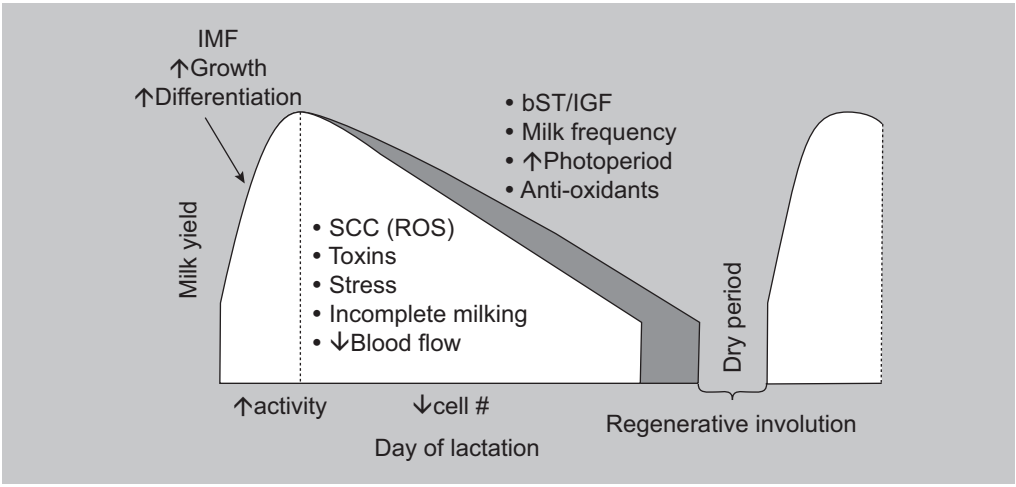

Figure 5. Diagrammatic representation of factors that influence persistency of lactation. Early lactation is characterized by an increase in secretory activity per cell. IMF may induce mammary proliferation or enhance mammary differentiation. Declining milk production is primarily due to a decline in the number of mammary secretory cells. Factors that increased cell proliferation or decrease apoptosis serve as enhancers of persistency, while the opposite is true for factors that decrease persistency (Source: Capuco et al.*, 2003).*

milking frequency (IMF) (5) anti-oxidants. Among factors that can increase apoptotic cell death and decrease persistency are: (1) mastitis –a number of stimuli may be involved including bacterial toxins, reactive oxygen species generated by phagocytic activity, damage due to neutrophil influx into the gland (2) neutrophil surveillance activity in the absence of infection (3) decreased milking frequency (4) decreased blood flow (5) stressors (6) pregnancy, in later stages of gestation this may negatively impact milk production due to competing nutrient partitioning demands and hormonally by apoptotic effects of estrogens. Evidence for the positive effectors of persistency, bST and IMF, will be described because of their relevance to subsequent discussion of dry period management.

Administration of bST has been shown to increase persistency of lactation in dairy cows and other ruminants (Phipps *et al.*, 1991; Van Amburgh *et al.*, 1997; Baldi, 1999; Bauman *et al.*, 1999). In light of the demonstration that the decline in milk yield with advancing lactation is primarily due to a decline in mammary cell number rather than cellular activity, it follows that the effect of bST on persistency is due to maintenance of the mammary cell population rather than maintenance of cellular secretory rate. Indeed, bST maintained cell number in lactating caprine mammary gland (Knight *et al.*, 1990) and data suggest that bST increases mammary cell proliferation during late gestation in sheep (Stelwagen *et al.*, 1993) and perhaps heifers (Stelwagen *et al.*, 1992). The influence of bST on the proliferative and apoptotic status of cells within the lactating bovine mammary gland has been evaluated (Capuco *et al.*, 2001a). The nuclear proliferation antigen Ki67 was used as an index for the relative proliferation state of mammary tissue. This protein serves as a marker for cells that are engaged in cell cycle progression, due to its presence during all phases of the cell cycle except the quiescent G_0 phase (Gerdes *et al.*, 1984) and its correlation with

cellular proliferation under a variety of physiological conditions in human, rodent and bovine tissues (Hardville and Henderson, 1966; Shayan *et al*., 1999; Capuco *et al*., 2001a). Treatment of first lactation dairy cows with bST for 7 d increased the proportion of cells expressing Ki67 threefold (Figure 6). The effect of bST was evident when cows were fed ad libitum (2.5 vs. 0.7%), or when they were restricted to 80% of ad libitum intake (0.8 vs. 0.3%) to induce a negative energy balance (Capuco *et al*., 2001b). Overall, cows in negative energy balance had a lower percentage of mammary cells expressing Ki67 than did comparable cows in positive energy balance. These data strongly support the hypothesis that bST increases cellular proliferation in the bovine mammary gland and that mammary cell proliferation is blunted by reduced energy balance. Additionally, these data suggest that a proliferative response to bST would be blunted during early lactation (Capuco *et al*., 2001a) due to the negative energy balance experienced by cows during early lactation.

Although treatment of lactating cows with bST apparently increased mammary cell proliferation (Capuco *et al*., 2001a), earlier investigations had indicated that bST treatment did not cause net growth of the lactating bovine mammary gland (Capuco *et al*., 1989; Binelli *et al*., 1995). The earlier results do not infer that bST does not increase the rate of cell proliferation, simply that the rate of cell proliferation did not exceed the rate of cell death. We suggest that treatment

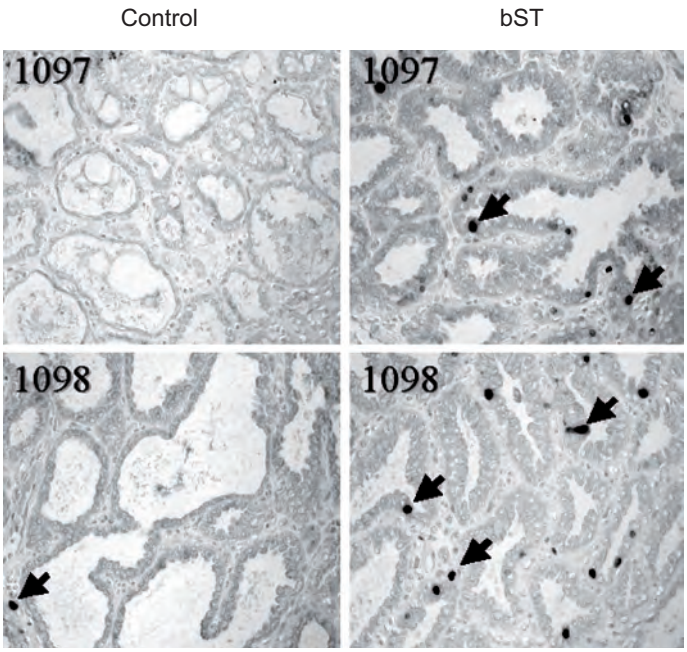

Figure 6. Effect of bST administration for 7 d on Ki67 labeling of mammary tissue from primiparous Holsteins. Tissues were taken by biopsy during control and bST treatment periods for each cow. Representative (dark) nuclei expressing Ki67 are indicated by arrows. Administration of bST increased the Ki67 labeling index, indicative of increased cell proliferation. The number within a panel is the cow identification number. Each cow was evaluated during a control and bST treatment period (Source: Capuco et al.*, 2001a).*

with bST increased the rate of mammary cell proliferation so as to reduce the rate of mammary regression during lactation. Additional study will be necessary to determine if extended treatment with bST increases the number of mammary cells relative to that of cohort controls. Previous slaughter studies may not have been sufficiently sensitive to detect small, e.g., 10%, differences in cell number. Alternatively, increased cell renewal may enable milk synthesis in bST-treated cows to continue at high capacity by enhanced replacement of senescent or resting or inactive secretory cells so that a greater proportion of cells secrete at high capacity. Support for these latter hypotheses will be provided in later sections.

Increased milking frequency at the beginning of lactation has been shown to increase milk yield not only during IMF but also after its cessation (Bar-Peled *et al.*, 1995). Using a within udder experimental design, increased milking frequency (4X) during mid lactation was shown to increase cell proliferation when assessed 4 wk after initiation of treatment (Hillerton *et al.*, 1990). We have recently evaluated the immediate effects of increased milking frequency initiated during early lactation on mammary growth and the long-term effects on milk yield (Hale *et al.*, 2003). A novel approach of utilizing four very uneven milking intervals was employed to obtain increased frequency without the added labor and other inherent costs involved in establishing a traditional pattern of increased milking frequency with fairly even milking intervals. Cows were divided into three treatment groups: 1) controls were cows milked twice daily (2X) beginning at parturition, 2) IMF1: cows milked four times daily (4X) from d 1 to 21 postpartum 3) IMF4: cows milked 2X from d 1 to 3 and 4X d 4 to 21 postpartum. The 4X cows were milked immediately before 2X cows and again (~3 h later) after the 2X cows at both morning and evening milkings. All cows were milked 2X from d 21 to 305 postpartum. Milking 4X during early lactation increased milk yield not only during the treatment period but overall elicited an 8% increase in milk yield for the entire lactation ($P < 0.05$). Mammary biopsies from four cows per treatment were obtained on d 7 and 14 postpartum to assess mammary cell proliferation. ^{3}H-thymidine incorporation by tissue slices was increased ($P = 0.09$) on d 7 in IMF1 cows, and arithmetic means for the percentage of cells expressing Ki67 proliferation antigen were consistent with a proliferative response to IMF. Increased milking frequency during early lactation may increase mammary growth and thus produce a carryover effect on milk production for the majority of lactation. A carryover effect was observed with a minimal increase in labor and operating costs and was effective when increased milking was initiated on the first day of lactation or on day 4, after the routine interval for discarding milk colostrum. Whether this protocol significantly increases milk component secretion remains to be demonstrated.

Other investigations had shown that increased milking frequency enhanced mammary cell proliferation. Increased milking frequency increased milk production of goats, due to a rapid increase in activity of mammary secretory cells, often followed by proliferation of secretory tissue (Knight *et al.*, 1990; Lin and Buttle, 1991). Increased milking frequency is hypothesized to increase milk production by lessening the accumulation of a feedback inhibition of milk secretion (Henderson and Peaker, 1984). Because frequent milking of one mammary gland has no effect on milk secretion by the opposing gland, it is clear that milk removal and not systemic effects of milking plays an important role in establishing secretion rate (Linzell and Peaker, 1971). Whether removal of inhibitors of lactation can adequately explain the impact of supplemental milkings with very short intermilking intervals is uncertain.

Another potential mechanism to explain an increase in milk yield that persists long after IMF imposed during early lactation is an effect on degree of cell differentiation. Prolactin is released at each milking and it has been shown to be critically important in promoting final stages of mammary epithelial cell differentiation in the final days of lactogenesis (Akers *et al*., 1981a; Akers *et al*., 1981b). Thus a prolactin-mediated enhancement of cell differentiation during early lactation may provide for an increased proportion of mammary epithelial cells that are fully secretory. The lactating mammary gland displays a degree of heterogeneity, with some alveoli fully functional and others apparently resting or involuting (Molenaar *et al*., 1992; Molenaar *et al*., 1996). A differentiation promoting effect of IMF may effectively decrease the number of cells that are inactive with respect to milk secretion. As discussed subsequently, the increased apoptotic rate during early lactation may aid in this process by supporting the removal of nonfunctional epithelial cells by apoptosis.

Population dynamics during a 60-day dry period

In contrast to other species, normal management of dairy cows and goats results in an overlap of lactation and pregnancy, such that these animals are typically pregnant when milking is terminated during late lactation. Thus, when milk stasis occurs, the mammogenic and lactogenic stimulation of pregnancy opposes stimuli for mammary involution. Processes of mammary growth and involution both occur during this dry period between successive lactations. Milk production efficiency can be increased by development and utilization of schemes that increase persistency of lactation and minimize the duration of the dry period.

Aspects of mammary growth during a traditional 60-day dry period have been investigated in comparison with cows that were milked during the prepartum period (Capuco *et al*., 1997). Multiparous Holstein cows were slaughtered at 7, 25, 40 and 53 days into the dry period (53, 35, 20 and 7 d prepartum) and total mammary DNA and ^{3}H-thymidine incorporation into mammary tissue slices was determined. There was no significant loss of mammary cells (DNA) during the dry period, and total number of mammary cells increased with advancing stages of the dry period. Total DNA did not differ between mammary glands of dry and lactating cows; however, increased DNA synthesis in dry cows indicated that replacement of mammary cells increased during the dry period. Autoradiographic localization of incorporated ^{3}H-thymidine indicated that the replicating cells were primarily (> 90%) epithelial. Thus, in cows the dry period may be important for replacing senescent cells prior to the next lactation. Furthermore, although cows appeared to enter the next lactation with the same number of mammary cells regardless of whether or not they had a dry period, a greater percentage of those cells were epithelial in cows that had a dry period.

Although extensive mammary cell loss does not occur during a typical bovine dry period, extensive tissue remodeling does occur and this includes changes in cell populations, alveolar structure and synthesis of extracellular matrix (Holst *et al*., 1987; Hurley, 1989; Capuco *et al*., 1997). Others have demonstrated that apoptotic cell death occurs after milk stasis in the bovine mammary gland (Quarrie *et al*., 1996; Wilde *et al*., 1997) and that increased milking frequency decreases apoptotic rate (Li *et al*., 1999a). Utilizing immunohistochemical techniques, we have recently demonstrated that there is an increase in both the apoptotic index and Ki67 labeling

index (Gerdes *et al*., 1983; Capuco *et al*., 2001a) within the first 10 d of the dry period (Figure 7). These results are consistent with the concept that there is extensive cell turnover during the dry period, as the processes of cell proliferation, assessed by Ki67 labeling index, and apoptotic cell death were simultaneously increased. In light of the continued increase in cell proliferation during the dry period, it is somewhat puzzling that cell proliferation, assessed by Ki67 labeling, declined between 10 and 21 d after milk stasis. It was at 21 d dry that increased DNA synthesis was first detected in the earlier study by Capuco *et al.* (1997).

Increased mammary cell turnover during the dry period is likely a consequence of concomitant pregnancy and milk stasis, as the mammogenic effects of pregnancy tend to counterbalance the apoptotic effects of milk stasis by promoting cell proliferation and inhibiting apoptosis. In this regard the events occurring during the dry period differ significantly from mammary involution as frequently studied in rodent models, wherein mammary involution is rapid and extensive. This is evident by comparing the maximal apoptotic indices obtained after milk stasis for cow (1.3% on d 10, Figure 7) with those obtained for mouse mammary gland following milk stasis (7.2% on d 3, Capuco *et al*., 2002). When mice are pregnant at the time of weaning, mammary apoptosis is reduced and cell proliferation enhanced relative to their nonpregnant counterparts (Capuco *et al*., 2002). Consequently, we have referred to the dry period as a period of "regenerative involution" to describe more fully the processes of cell renewal and tissue remodeling that occur following milk stasis with concomitant pregnancy (Capuco *et al*., 2003).

We have hypothesized that the dry period may be critical for replacing progenitor cells that are responsible for expanding and maintaining the number of mammary secretory cells (Capuco and Akers, 1999). This concept is supported by two current studies. In one study, the percentage of pale cells in the mouse mammary gland were shown to decline from peak lactation to extended

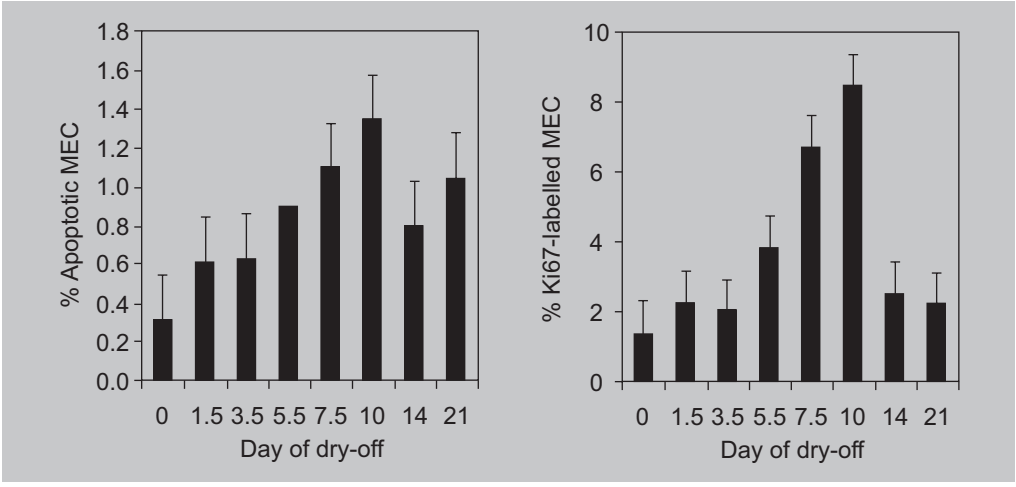

Figure 7. Relative rates of apoptosis and cell proliferation during the dry period. Apoptosis was assessed by TUNEL assay and proliferation by expression of Ki67 antigen (Source: Annen, Capuco and Collier, unpublished data).

lactation (Capuco, Hadsell and Ellis, unpublished data). In a second ongoing study, expression of genes that may serve as markers for bovine mammary progenitors (e.g., telomerase and ATP binding cassette multidrug resistance transporter) were assessed by real-time RT-PCR and found to increase during the dry period (Capuco, unpublished data). As further support, is the demonstration that mammary glands of rats that were not permitted a dry period had fewer cells at midlactation than did glands of rats that were permitted a dry period of optimal length; although cell number did not differ at onset of the lactation (Paape and Tucker, 1969). If replacement of senescent cells is a critical event during the dry period, one would hypothesize that without a dry period of sufficient length to allow replacement of senescent cells, persistency of the ensuing lactation will be decreased.

In contrast to cows, a dry period may not be necessary for optimal milk production in dairy goats. Two investigations have addressed the importance of a dry period in goats. In the first (Knight and Wilde, 1988), lactating goats were induced into ovulation and mated out of season. The goats entered the next lactation without a dry period and milk production was found to be 12% less than the previous lactation. However, seasonal effects on lactation may have confounded the results. Subsequently, Fowler *et al*. investigated the necessity for a dry period using a within animal, half-udder, design (Fowler *et al*., 1991). One gland was milked during the prepartum period while the other was dried off 24 weeks (170 d) prior to parturition. There was no difference in milk production between glands or a trend for improved production in the dried gland. These data suggest that a dry period is not necessary for optimizing milk production in the next lactation in goats. Because significant mammary growth occurs during early lactation of goats and milking induces the release of somatotropin in goats, but not cows, the mammary gland of goats may demonstrate a greater capacity to continue cell proliferation and renewal processes, such as those detected during a bovine dry period, into early stages of lactation. This may influence the necessity for a dry period in this species.

A dry period between lactations has been a standard in dairy management practices since the 1800's, with durations of 10 d to 10 wks (Dix Arnold and Becker, 1936). In the early 1900's, an 8-wk dry period became the most widely used because it was believed to optimize productivity in the subsequent lactation (Dix Arnold and Becker, 1936). During World War II, the 305-d lactation and 60-d dry period was adopted to provide for maximal milk production during a time of food shortages and maintenance of genetic progress (Knight, 1998) and remains the current standard. There is ample evidence from studies utilizing a variety of approaches to indicate that in the absence of a dry period, milk production is reduced in the subsequent lactation (Swanson, 1965) and that a dry period is necessary for reasons that center on the mammary gland rather than the nutritional status of the animal (Smith *et al*., 1967). Recommendations for dry period length have been based primarily on retrospective studies (Coppock *et al*., 1974) and experiments (Swanson, 1965; Sørensen and Enevoldsen, 1991) utilizing cows that were not the high milk producers prevalent in today's production systems (for reviews see: Bachman and Schairer, 2003; Grummer and Rastani, 2004; Annen *et al*., 2004a). Recently, Bachman and colleagues have evaluated the feasibility of accelerating mammary involution during the dry period by administration of estradiol-17β cipionate at dry off (Athie *et al*., 1996; Athie *et al*., 1997). By accelerating the early stages of involution, it was hoped that dry periods <40 d could be employed without a loss of milk production in the subsequent lactation. Even in the absence of estrogen treatment, no milk

deficit or added management or health costs were incurred by utilizing a dry period of 34 vs. 59 d (Bachman, 2002). This study emphasizes the importance for reevaluating the "optimum" length utilizing today's high-production cows and current management systems, including the use of bST, photoperiod and increased milking frequency. As opposed to the situation in many of the seminal studies upon which dry period recommendations were based, improvement in animal genetics and management have resulted in lactations that could easily extend beyond 305 d and cows that are producing copious quantities of milk 60 d before expected parturition.

Adoption of the most appropriate dry period length for a particular production system is an economic decision and should be based upon a variety of factors in addition to the balance of forfeited milk during the dry period and enhanced milk production in the ensuing lactation. The impact of dry period length upon other factors such as feeding management, animal health, animal housing and periparturient care, all within particular management systems, should be weighed in conjunction with effects on milk revenue (yield and composition) and colostrum quality (Bachman and Schairer, 2003; Annen *et al*., 2004a; Grummer and Rastani, 2004). A combination of basic and applied science is necessary to understand the biology underlying the relationship between dry period length and milk production, and to evaluate the many interactions with management systems.

Implications of cell turnover to shortened dry periods

Based upon the concept that a dry period is necessary for restoration of the full compliment of mammary secretory cells and replacement of senescent secretory cells and progenitor cells, it is reasonable to assume that management procedures that influences cell loss and cell turnover can impact the magnitude and necessary duration of cellular events that occur during the dry period. Promotion of cell turnover and lactational persistency during the current lactation may lessen the demands for a dry period. Conversely, promotion of cell growth and turnover during early phases of the ensuing lactation may alleviate the negative impact of a dry period of insufficient duration. Furthermore, the quantity of milk being produced by the mammary gland at dry-off will likely impact the speed and magnitude of events that occur during the dry period. It is imperative that the impact of management practices that can potentially alter cell turnover be evaluated with regard to impact on cell kinetics during the dry period and subsequent milk production.

A recent study evaluated milk production effects of shortened or omitted dry periods for cows at mature-equivalent production > 12,000 kg of milk and treated with bST (Annen *et al*., 2004b). The study used 2 commercial dairy herds and the University of Arizona dairy herd and included 4 treatments. Five multiparous and 5 primiparous cows from each farm were assigned to each treatment; 1) 60-d dry period, label use of bST (60DD), 2) 30-d dry period, label use of bST (30DD), 3) continuous milking, label use of bST (CMLST) and 4) continuous milking with continuous use of bST (CMCST). Per label, bST use started at 57 to 70 DIM and ended 14 d before drying (60 and 30DD) or expected calving date (CMLST) and then resumed 57 to 70 DIM in the subsequent lactation. CMCST cows received bST on a 14 d schedule through parturition and into the next lactation. For multiparous cows, average milk yields in the ensuing lactation were not significantly impacted ($P > 0.05$) by shortened or omitted dry periods with either bST protocol, as compared with cows conventionally managed for a 60-d dry period. Milk yields for the first

17 wk of lactation for multiparous cows in 30DD, CMLST, and CMCST vs. 60DD were 46.6, 43.4, 46.5, and 47.7 ± 2.1kg/d, respectively (Figure 8). However, for primiparous cows, average milk yields were reduced ($P < 0.001$) by continuous milking and shortened dry periods. Milk yields for the first 17 wk of lactation for cows in 30DD, CMLST, and CMCST vs. 60DD were 38.3, 35.1, and 37.5 vs. 44.1 ± 1.3 kg/d, respectively (Figure 8). These data for cows that were treated with bST differ markedly from previous studies demonstrating 20 – 25% reductions in milk yield in cows managed without a dry period (Swanson, 1965). Treatment with bST may enhance turnover of mammary cells (Capuco *et al*., 2001a) and reduce or eliminate the dependence on such processes during the dry period of multiparous cows. For primiparous cows, mammary cell number is not only restored, during the dry period, to that present at the beginning of first lactation, but additional growth occurs. A shortened or omitted dry period may impede the supplemental mammary growth that occurs during the dry period of primiparous cows, resulting in fewer secretory cells and reduced milk yield in the subsequent lactation as compared with conventionally managed cohorts.

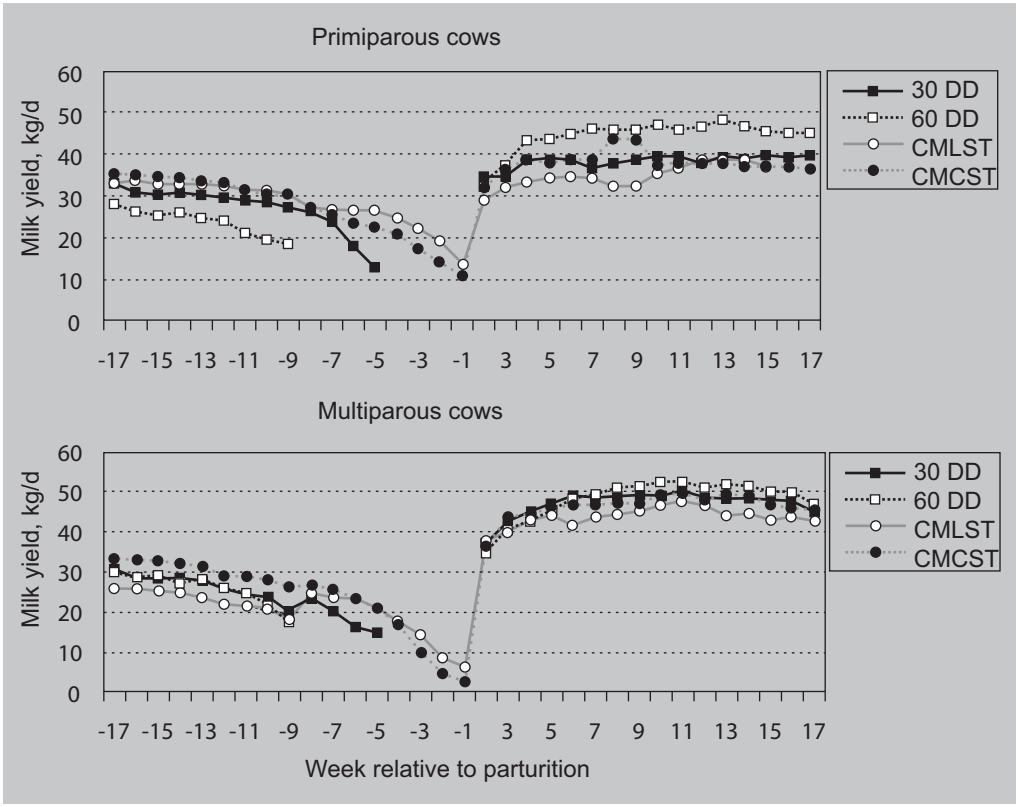

Figure 8. Milk yield during late gestation and 17 wk of the next lactation in cows subjected to 30 day (30DD), 60 day (60DD) or omitted (CMLST) dry periods (all in combination with label bST supplementation) or omited dry period with continuous bST supplementation (CST). See text for further explanation (Source: Annen et al*., 2004b).*

For multiparous cows, milk composition was unaffected by treatment; whereas for primiparous cows, milk protein percentage was significantly increased by bST and 30DD compared with the 60DD. Milk protein percentages for primiparous cows were 3.16, 3.04, 3.06 and 2.85 for 30DD, CMLST, and CMCST vs. 60DD, respectively. Quality aspects of prepartum milk and colostrum require additional characterization. Very low milk yields in some continuously milked cows immediately before parturition may result in a secretion that is more similar to dry secretions than milk. These changes may alter the appearance and consistency of milk and may also result in cows being identified as having mastitis. These changes in the physical properties and composition of prepartum mammary secretions also raise questions of salable milk and warrant further research.

For multiparous cows, milk income generated for short dry periods or for continuous milking might increase the dairy producer's profitability. At 17 wk of the subsequent lactation, estimates of the cumulative net margins of multiparous cows in the 30DD and continuous milking treatments exceeded those of 60DD cows by $40 to $60 per cow (Annen *et al.*, 2004b).

Primiparous cows are more sensitive than multiparous cows to the negative impact of continuous milking (CM) or shortened dry period on subsequent milk production. Primiparous Holsteins were used to evaluate effects of continuous milking (CM) combined with bST and increased milking frequency (IMF) in two, simultaneous experiments conducted at the University of Arizona. Both studies utilized a half-udder model. The first study was designed to evaluate effects of bST and CM on milk production and MEC turnover during late gestation and early lactation (E.L. Annen, A.C. Fitzgerald and R.J. Collier, unpublished). Eight cows were used: four were bST supplemented (+bST) throughout the experiment and four were not treated with bST (-bST). Within animal, one udder half was milked continuously throughout late gestation (CM) and the other udder half was dried 60 d prior to expected parturition (CTL). The experiment was conducted during the last 60 d of gestation and first 30 d of the subsequent lactation, and all cows were milked twice daily (2X) both pre- and postpartum. Tissues were obtained by mammary biopsy. Proliferation was assessed by Ki67 labeling and apoptosis by TUNEL assay. MEC proliferation was affected by the interaction of dry period lactation status (CTL vs. CM) by time, but was unaltered by bST treatment (Figure 9A). In CTL udder halves, MEC proliferation peaked at d –8 with 4.5% of MEC expressing the Ki67 antigen. At this time, MEC proliferation was 50% greater in CTL halves than CM halves. In CM udder halves, MEC proliferation was highest at d –20. Similarly, Capuco *et al.* (1997) demonstrated that ^{3}H-thymidine incorporation in CM glands was 30 – 50% of that for CTL quarters during the period from d –21 to –7 prepartum. After parturition, the percentage proliferating MEC decreased and was not influenced by lactation status of the udder half during late gestation. Low levels of proliferation in the mammary gland following MEC differentiation and onset of copious secretion might be expected. Similar Ki67 labeling indices were reported for early lactation (Capuco *et al.*, 2001a) and in vitro studies demonstrated that culturing MEC in lactogenic media caused a decline in number of pale-staining cells (Smith and Medina, 1988). A reduction in pale cells is associated with reduced mammary proliferation in cattle (Ellis and Capuco, 2002).

Apoptosis of MEC was altered by time and the interaction of time by dry period lactation status, but was not affected by bST treatment (Figure 9B). In CTL udder halves, MEC apoptosis

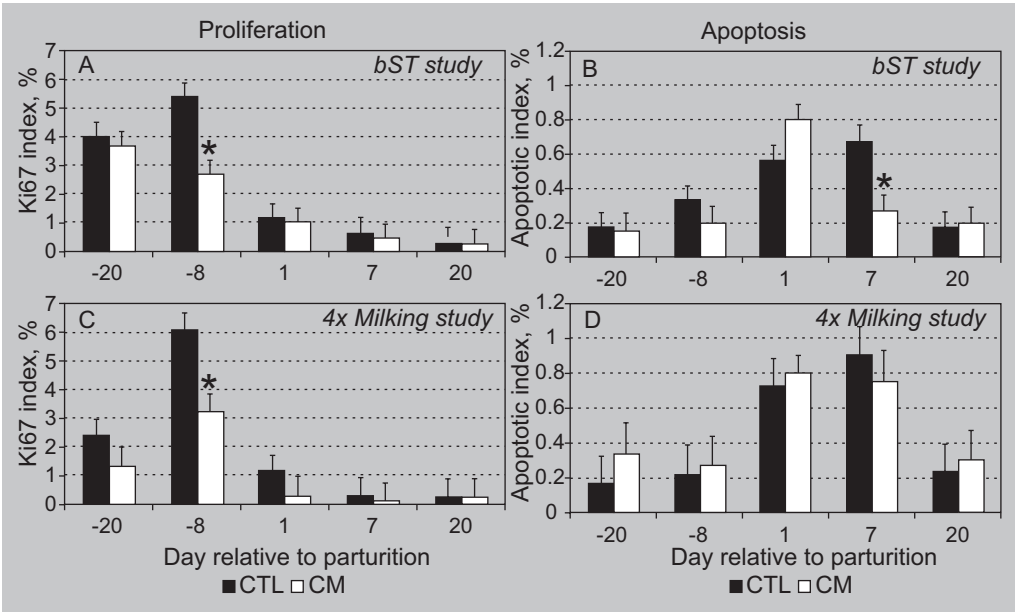

Figure 9. Changes in mammary epithelial cell proliferation and apoptosis during late-gestation and early lactation in control (60-d dry, CTL) and continuously milked (CM) udder halves milked twice or four times daily during early lactation.

No effect of bST was detected for Ki67 antigen index or apoptotic index ($P > 0.05$) so cell means are not shown. Postpartum Ki67 antigen index was not altered by milking frequency, but the apoptotic index tended ($P < 0.07$) to be increased by 4X milking. Differences between treatments are indicated by an asterisk ($P < 0.05$) (Source: Annen et al. *(unpublished data) and Fitzgerald* et al. *(unpublished data))*

increased during late gestation into early lactation and peaked 7 d postpartum. In CM udder halves, MEC apoptosis peaked at d 2, but was unchanged at all other time points. Between dry period treatments, MEC apoptosis was greater in CTL glands at d 7 (Figure 9B). Furthermore, CTL and CM halves differed in the temporal pattern of apoptosis during late gestation and early lactation. In CTL halves, apoptosis increased throughout late gestation into early lactation until d7, then decreased rapidly, whereas CM halves showed only a spike in MEC apoptosis at d 2. These results are consistent with greater apoptosis and cell turnover in mammary glands permitted a dry period than in those that are continuously milked. Three hypotheses have been proposed to explain increased apoptosis during early lactation. They include: 1) an increase in apoptotic leukocytes that have migrated into the mammary epithelium and are indistinguishable from MEC due to morphological changes during apoptosis (Capuco *et al.*, 2001a), 2) removal of old or dormant MEC from the mammary epithelium replacing them with new MEC that were generated during the late gestation, and 3) elimination of new cells that were generated during late gestation, but did not undergo differentiation during the final stage of lactogenesis (Sørensen and Sejrsen, 2003). In CM udder halves, a rapid decline in apoptosis after d 2 combined with reduced MEC proliferation at d –7 suggests that an early decrease in apoptosis compared to CTL halves was required to keep more old cells in the mammary epithelium to maintain

total cell numbers. Total cell number, as measured by total parenchyma DNA, is not altered by lactation status during late gestation (Swanson *et al*., 1967; Capuco *et al*., 1997). A reduction in proliferation accompanied by a reduction in apoptosis to maintain total MEC numbers, causes an increased proportion of old MEC in CM glands (Capuco *et al*., 1997; Annen *et al*., 2004b). The extent of apoptosis during early lactation may reflect the number of new (replacement) MEC generated during late gestation. It remains to be determined if the carryover of old MEC into the next lactation impacts the persistency of the lactation. Histological evaluation of tissues from CTL and CM glands supports the concept that a dry period is important for generating fully functional mammary epithelial cells without a significant population of resting or inactive cells. After parturition, there was a gradual decline in the number of resting cells in CTL glands so that by last sampling at 20 d postpartum the vast majority of cells were fully secretory. In contrast at 20 d postpartum, CM glands contained many cells that were relatively inactive in appearance (Figure 10).

Given the high between and within animal variability in milk production data, a true evaluation of production parameters would require more animals and production data beyond early lactation. Prepartum half-udder milk yield was only numerically increased by bST treatment (Figure 11A). Spontaneous dry-off during final days of gestation was not affected by bST treatment. The +bST,

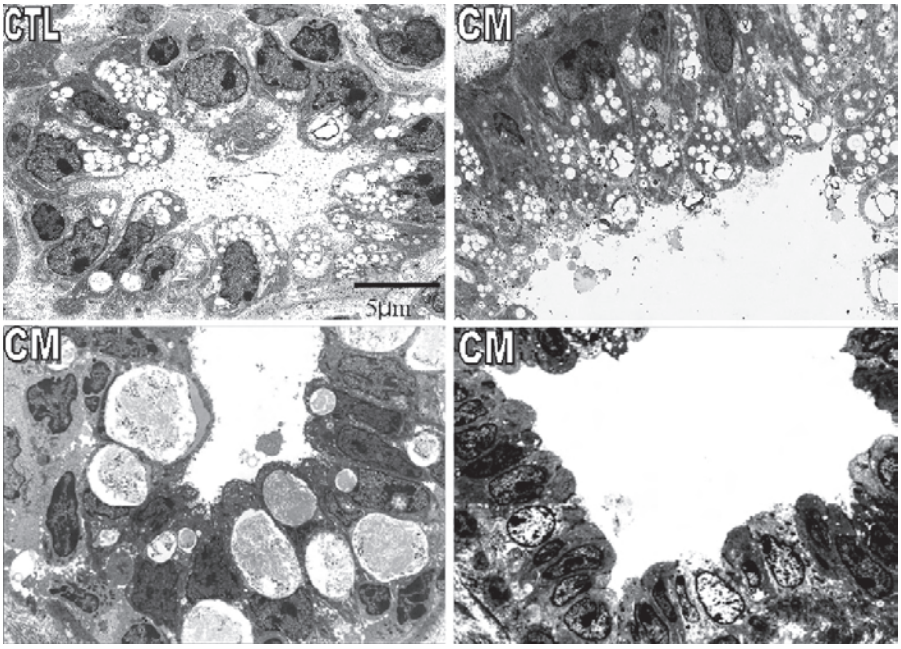

Figure 10. Increased presence of inactive mammary epithelial cells in continuously milked (CM) mammary glands at d 20 of lactation. Electron micrographs of mammary tissue from control quarters (CTL) and CM quarters show a fully differentiated phenotype in tissues from CTL but a range of phenotypes from fully differentiated (upper right) to resting (lower right) or potentially involuting (lower left) mammary epithelial cells.

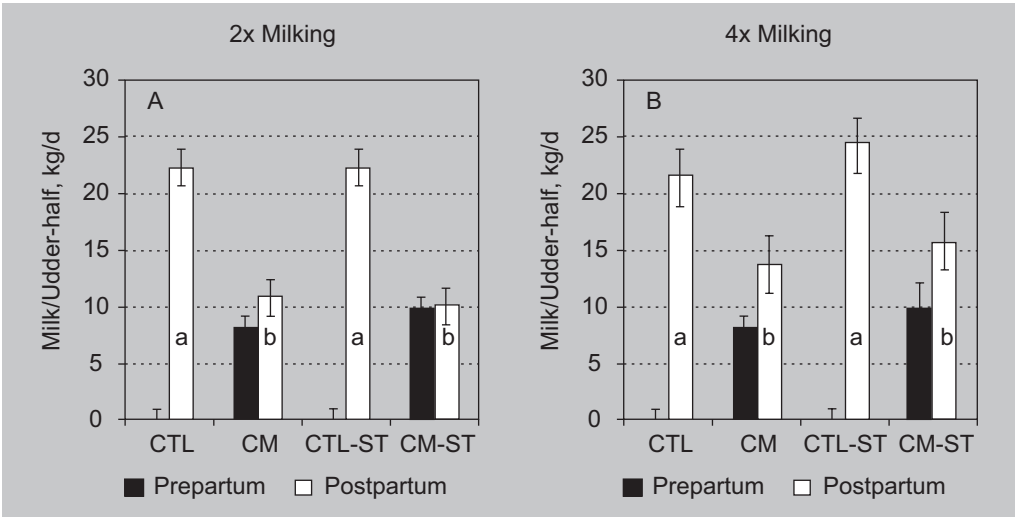

Figure 11. Milk yield in cows with or without bST supplementation and continuously milked (CM) or 60-d dry (CTL) udder halves.
Treatments without common letter differ (P < 0.05). Means were adjusted using data collected from d 67 to 61 prepartum as a covariate.

CM halves averaged 5.6 d dry and –bST, CM halves averaged 3.1 d dry. Postpartum half-udder milk yield was dramatically reduced in CM halves compared to CTL halves, regardless of bST treatment. Cow numbers were adequate to detect a decrease of this magnitude. The fact that a milk yield increase was not detected in +bST, CTL or +bST, CM udder halves during early lactation may have been influenced by small cow numbers and the fact that the study was carried out during a part of the lactation curve that is already increasing. Negative energy balance dynamics during early lactation also may have limited the milk yield response to bST. Diminished responses to bST have been demonstrated in early lactation cows (Vicini *et al*., 1991) and energy restricted cows (McGuire *et al*., 1995).

The second half-udder study was identical to the first study described above, except eight primiparous, Holstein cows were milked 2X until parturition, then milked four times daily (4X) for the first 30 d of the subsequent lactation. Early-lactation IMF may be an approach to enhance mammary growth and differentiation and to minimize or recoup milk losses observed in primiparous cows. Thus, the second experiment was formulated to determine the effects of bST and IMF on MEC proliferation and apoptosis in CM and CTL udder halves (A.C. Fitzgerald, E.L. Annen and R.J. Collier, unpublished). Within 4X cows there was a significant effect of time on cell proliferation, but no significant effect of dry period lactation status, or lactation status by day interaction (Figure 9C). However, the data show numerical reductions in proliferation in CM halves at d –20 and –8 compared to CTL halves. These observations agree with measures of MEC proliferation in lactating and dry glands at d –21 and –7 reported by Capuco et al. (1997). In contrast to observations in 2X cows described above, the Ki67 index in both 4X, CTL and 4X, CM halves is similar to ^{3}H-thymidine incorporation index measured by Capuco et al.

(1997). Because the 4X milking was not applied until after parturition, prepartum treatments were identical between the studies, suggesting that the difference at –20d was due to random effects. Together these studies clearly show diminished cell proliferation in CM quarters during late gestation. Postpartum MEC proliferation was not altered by 4X milking frequency or CM, but was reduced compared to prepartum proliferation indexes (Figure 9C). Together, these studies do not support the hypothesis (Hale et al., 2003) that 4X milking during early lactation enhances cell proliferation (Figure 9A vs. 9C). Although this cannot be totally ruled out because Ki67 labeling measures the percentage of cells cycling rather than the transit time (rate) in the cell cycle, the data suggest that IMF enhances milk yield through mechanisms other than increased MEC proliferation. Effects on cell differentiation may be a more likely mechanism.

Apoptosis in the mammary epithelium has been shown to increase at dry-off (Wilde *et al*., 1997; Capuco and Akers, 1999; Wilde *et al*., 1999) and with less frequent milking in goat half-udder studies (Li *et al*., 1999a). Therefore, higher apoptotic indices were expected during lactation in 2X than 4X mammary glands independent of dry period lactation status. However, consistent with previous results (Hale *et al*., 2003), postpartum apoptotic indexes were higher in 4X than 2X cows (Figure 9B vs. 9D). Hale *et al.* (2003) hypothesized that increased apoptosis in early lactation was caused by apoptotic leukocytes in the mammary epithelium as a result of tissue edema. Because leukocytes cannot be distinguished from MEC during advanced stages of apoptosis they could be erroneously included in apoptotic indexes for MEC. However, in this study there were no visible differences in udder edema in 4X vs. 2X cows during the first week postpartum. As suggested earlier, increased apoptosis during early lactation may provide a mechanism for removal of senescent cells and may be directly correlated with productivity of the mammary gland. If increasing milking frequency stimulates a 15 to 25% increase in milk yield (Bar-Peled *et al*., 1995; Hale *et al*., 2003), then shedding old MEC and replacing them with new would support additional milk synthesis. However, in the current study, increased MEC proliferation apparently did not accompany increased apoptosis. We hypothesize that high daily rates of apoptosis in 4X cows during early lactation are required for removal of old or dormant MEC to enable hypertrophy (rather than hyperplasia) and enhanced synthetic capacity of MEC.

As mentioned previously for the 2X, half-udder study, more animal numbers are required for an accurate assessment of the effects of bST and IMF on CTL and CM half-udder milk yield. Production data for 4X cows are presented in Figure 11B. Prepartum treatment of 2X and 4X cows did not differ, as all cows were milked 2X until parturition. Therefore, prepartum milk yields are similar for both experiments (Figures 11A and B). Similar to the 2X study, milk yield was dramatically reduced in 4X,CM udder-halves. Neither 4X milking nor bST enhanced milk yield in CTL or CM udder halves, although arithmetic differences were observed between 4X, CM and 2X, CM halves with or without bST.

These udder-half studies demonstrate several aspects about the relationship between milk production and cell kinetics during late gestation and early lactation and raise additional questions for investigation. The studies clearly demonstrate that there is greater cell turnover during the prepartum period in mammary glands that are permitted a traditional 60-d dry period than in those that are continuously milked. An effect of bST on cell proliferation and death during late gestation and early lactation could not be demonstrated, nor could an impact on milk

production be shown. The latter is consistent with previous literature. The studies also suggest that mechanisms such as increased differentiation during early lactation, rather than increased cell proliferation, are responsible for persistent milk production increases induced by IMF during early lactation. Worthy of additional investigation is the general correspondence between apoptotic frequency during early lactation and milk yield. CM mammary glands demonstrated lower milk production and apoptotic frequency than glands permitted a conventional dry period, 2X milked glands showed lower milk yield and apoptotic frequency than 4X glands and finally control and bST-treataed cows did not differ from controls with regard to frequency of mammary apoptosis during early lactation or milk production. Although greater animal number is required to fully interpret impact of treatment on milk production, these effects are intriguing. The studies emphasize the importance of the dry period and events occurring during early lactation upon lactational productivity.

Conclusions and perspectives

Data continue to accumulate that support a central role for mammary cell turnover in promoting lactational productivity. During lactation, cell turnover provides for the replacement of cells possessing sub optimal capacity and thus can promote the maintenance of the secretory capacity per cell. Small adjustments in rates of cell proliferation and/or apoptosis can have significant impact upon lactational persistency. A number of current management tools (bST, photoperiod, IMF) may operate in part through effects upon processes of cell proliferation and death. During the dry period cell turnover appears critical for promoting maximal milk yield in the following lactation. This may serve to remove senescent cells and to provide for a more homogeneously and uniformly secretory epithelium. The hypothesis that turnover of mammary progenitor cells is a key event during the dry period is currently under investigation. Additional tools to permit thorough evaluation of kinetics of progenitor cells will aid this quest. In addition to events occurring during the dry period, the increased rate of MEC apoptosis during early lactation may be important for transitioning from a dry period, or lactation, to a maximally functional subsequent lactation. Gene expression studies during this period may be particularly informative. Others have shown that it is feasible to decrease dry period length to 30 d without reducing subsequent milk yield. Additional studies are needed utilizing cows with genetic potential relevant to today's dairy industry to evaluate the impact of management on optimal dry period length, the necessity for a dry period, and mechanisms to overcome deficits induced by insufficient dry period management. Dairy heifers are particularly sensitive to shortcomings in dry cow management because of their necessity for continued mammary and body growth. Treatment of multiparous cows with bST may be economically feasible for promoting a persistent lactation and reducing or eliminating the need for a dry period. Because of the potential impact of dry period management on lactational persistency it is important that whole lactation data be used for weighing the efficacy of various procedures.

References

Akers, R.M., D.E. Bauman, A.V. Capuco, G.T. Goodman & H.A. Tucker (1981a). Prolactin regulation of milk secretion and biochemical differentiation of mammary epithelial cells in periparturient cows. Endocrinology, 109, 23-30.

Akers, R.M., D.E. Bauman, G.T. Goodman, A.V. Capuco & H.A. Tucker (1981b). Prolactin regulation of cytological differentiation of mammary epithelial cells in periparturient cows. Endocrinology, 109, 31-40.

Annen, E.L., R.J. Collier, M.A. McGuire & J.L. Vicini (2004a). Effects of dry period length on milk yield and mammary epithelial cells. Journal of Dairy Science, 87 Suppl E, E66-E76.

Annen, E.L., R.J. Collier, M.A. McGuire, J.L. Vicini, J.M. Ballam & M.J. Lormore (2004b). Effect of modified dry period lengths and somatotropin on yield and composition of milk from dairy cows. Journal of Dairy Science, 87, 3746-3761.

Athie, F., K.C. Bachman, H.H. Head, M.J. Hayen & C.J. Wilcox (1996). Estrogen administered at final milk removal accelerates involution of bovine mammary gland. Journal of Dairy Science, 79, 220-226.

Athie, F., K.C. Bachman, H.H. Head, M.J. Hayen & C.J. Wilcox (1997). Milk plasmin during bovine mammary involution that has been accelerated by estrogen. Journal of Dairy Science, 80, 1561-1568.

Bachman, K.C. (2002). Milk production of dairy cows treated with estrogen at the onset of a short dry period. Journal of Dairy Science, 85, 797-803.

Bachman, K.C. & M.L. Schairer (2003). Invited review: bovine studies on optimal lengths of dry periods. Journal of Dairy Science, 86, 3027-37.

Baldi, A. (1999). Manipulation of milk production and quality by use of somatotropin in dairy ruminants other than cow. Domestic Animal Endocrinology, 17, 131-137.

Bar-Peled, U., E. Maltz, I. Bruckental, Y. Folman, Y. Kali, H. Gacitua, A.R. Lehrer, C.H. Knight, B. Robinzon & H. Voet *et al*. (1995). Relationship between frequent milking or suckling in early lactation and milk production of high producing dairy cows. Journal of Dairy Science, 78, 2726-2736.

Bauman, D.E., R.W. Everett, W.H. Weiland & R.J. Collier (1999). Production responses to bovine somatotropin in northeast dairy herds. Journal of Dairy Science, 82, 2564-2573.

Binelli, M., W.K. Vanderkooi, L.T. Chapin, M.J. Vandehaar, J.D. Turner, W.M. Moseley & H.A. Tucker (1995). Comparison of growth hormone-releasing factor and somatotropin: body growth and lactation of primiparous cows. Journal of Dairy Science, 78, 2129-2139.

Borst, D.W. & W.B. Mahoney (1980). Diurnal changes in mouse mammary gland DNA synthesis. Journal of Experimental Zoology, 214, 215-218.

Bursch, W., S. Paffe, B. Putz, G. Barthel & R. Schulte-Hermann (1990). Determination of the length of the histological stages of apoptosis in normal liver and in altered hepatic foci of rats. Carcinogenesis, 11, 847-853.

Capuco, A.V. & R.M. Akers (1999). Mammary involution in dairy animals. Journal of Mammary Gland Biology and Neoplasia, 4, 137-144.

Capuco, A.V., R.M. Akers & J.J. Smith (1997). Mammary growth in Holstein cows during the dry period: quantification of nucleic acids and histology. Journal of Dairy Science, 80, 477-487.

Capuco, A.V., S.E. Ellis, S.A. Hale, E. Long, R.A. Erdman, X. Zhao & M.J. Paape (2003). Lactation persistency: insights from mammary cell proliferation studies. Journal of Animal Science, 81 Suppl 3, 18-31.

Capuco, A.V., J.E. Keys & J.J. Smith (1989). Somatotrophin increases thyroxine-5'-monodeidonase activity in lactating mammary tissue of the cow. Journal of Endocrinology, 121, 205-211.

Capuco, A.V., M. Li, E. Long, S. Ren, K.S. Hruska, K. Schorr & P.A. Furth (2002). Concurrent pregnancy retards mammary involution: effects on apoptosis and proliferation of the mammary epithelium after forced weaning of mice. Biology of Reproduction, 66, 1471-1476.

Capuco, A.V., D.L. Wood, R. Baldwin, K. McLeod & M.J. Paape (2001a). Mammary cell number, proliferation, and apoptosis during a bovine lactation: relation to milk production and effect of bST. Journal of Dairy Science, 84, 2177-2187.

Capuco, A.V., D.L. Wood, T.H. Elsasser, S. Kahl, R.A. Erdman, C.P. Van Tassell, A. Lefcourt & L.S. Piperova (2001b). Effect of somatotropin on thyroid hormones and cytokines in lactating dairy cows during ad libitum and restricted feed intake. Journal of Dairy Science, 84, 2430-2439.

Chepko, G. & G.H. Smith (1997). Three division-competent, structurally-distinct cell populations contribute to murine mammary epithelial renewal. Tissue Cell, 29, 239-253.

Chepko, G. & G.H. Smith (1999). Mammary epithelial stem cells: our current understanding. Journal of Mammary Gland Biology and Neoplasia, 4, 35-52.

Coppock, C.E., R.W. Everett, R.P. Natzke & H.R. Ainslie (1974). Effect of dry period length on Holstein milk production and selected disorders at parturition. Journal of Dairy Science, 57, 712-718.

DeOme, K.B., L.J.Jr. Faulkin, H.A. Bern & P.B. Blair (1959). Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Research, 19, 515-520.

Dix Arnold, P.T. & R.B. Becker (1936). Influence of preceding dry period and of mineral supplementation on lactation. Journal of Dairy Science, 19, 257-266.

Ellis, S. & A.V. Capuco (2002). Cell proliferation in bovine mammary epithelium: identification of the primary proliferative cell population. Tissue and Cell, 34, 21-28.

Ellis, S., S. Purup, K. Sejrsen & R.M. Akers (2000). Growth and morphogenesis of epithelial cell organoids from peripheral and medial mammary parenchyma of prepubertal heifers. Journal of Dairy Science, 83, 952-961.

Ferguson, D.J. (1985). Ultrastructural characterisation of the proliferative (stem?) cells within the parenchyma of the normal "resting" breast. Virchows Archive A: Pathological Anatomy and Histopathology, 407, 379-385.

Fowler, P.A., C.H. Knight & M.A. Foster (1991). Omitting the dry period between lactations does not reduce subsequent milk production in goats. Journal of Dairy Research, 58, 13-19.

Gerdes, J., H.B.H. Lemke, H.H. Wacker, U. Schwab & H. Stein (1984). Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. Journal of Immunology, 133, 1710-1715.

Gerdes, J., U. Schwab, H. Lemke & H. Stein (1983). Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. International Journal of Cancer, 31, 13-20.

Grummer, R.R. & R.R. Rastani (2004). Why reevaluate dry period length. Journal of Dairy Science, 87, E77-85E.

Gulay, M.S., M.J. Hayen, K.C. Bachman, T. Belloso, M. Liboni & H.H. Head (2003). Milk production and feed intake of Holstein cows given short (30-d) or normal (60-d) dry periods. Journal of Dairy Science, 86, 2030-2038.

Hale, S.A., A.V. Capuco & R.A. Erdman (2003). Milk yield and mammary growth effects due to increased milking frequency during early lactation. Journal of Dairy Science, 86, 2061-2071.

Hardville, D.A. & C.R. Henderson (1966). Interrelationships among age, body weight, and production traits during first lactation of dairy cattle. Journal of Dairy Science, 49, 1254-1261.

Henderson, A.J. & M. Peaker (1984). Feed-back control of milk secretion in the goat by a chemical in milk. Journal of Physiology, 351, 39-45.

Hillerton, J.E., C.H. Knight, A. Turvey, S.D. Wheatley & C.J. Wilde (1990). Milk yield and mammary function in dairy cows milked four times daily. Journal Dairy Research, 57, 285-294.

Hogg, N.A., C.J. Harrison & C. Tickle (1983). Lumen formation in the developing mouse mammary gland. Journal of Embryology and Experimental Morphology, 73, 39-57.

Holst, B.D., W.L. Hurley & D.R. Nelson (1987). Involution of the bovine mammary gland: histological and ultrastructural changes. Journal of Dairy Science, 70, 935-944.

Hurley, W.L. (1989). Mammary gland function during involution. Journal of Dairy Science, 72, 1637-1646.

Knight, C.H. (1998). Extended lactation. Hannah Research Institute Yearbook, pp.30-39.

Knight, C.H., P.A. Fowler & C.J. Wilde (1990). Galactopoietic and mammogenic effects of long-term treatment with bovine growth hormone and thrice daily milking in goats. Journal of Endocrinology, 127, 129-138.

Knight, C.H. & M. Peaker (1984). Mammary development and regression during lactation in goats in relation to milk secretion. Quarterly Journal of Experimental Physiology, 69, 331-338.

Knight, C.H. & C.J. Wilde (1988). Milk production in concurrently pregnant and lactating goats mated out of season. Journal of Dairy Research, 55, 487-493.

Kordon, E.C. & GH. Smith (1998). An entire functional mammary gland may comprise the progeny from a single cell. Development, 125, 1921-1930.

Li, P., P.S. Rudland, D.G. Fernig, L.M. Finch & C.J. Wilde (1999a). Modulation of mammary development and programmed cell death by the frequency of milk removal in lactating goats. Journal of Physiology, 519 Pt 3, 885-900.

Li, P., C.J. Wilde, L.M. Finch, D.G. Fernig & P.S. Rudland (1999b). Identification of cell types in the developing goat mammary gland. Histochemical Journal, 31, 379-393.

Lin, C.L. & H.L. Buttle (1991). Effect of oestradiol benzoate and tamoxifen on the growth of and induction of progesterone receptors in the uterus and mammary gland of immature pigs. Journal of Endocrinology, 130, 259-265.

Linzell, J.L. & M. Peaker (1971). The effects of oxytocin and milk removal on milk secretion in the goat. Journal of Physiology, 216, 717-734.

McGuire, M.A., D.E. Bauman, D.A. Dwyer & W.S. Cohick (1995). Nutritional modulation of the somatotropin/insulin-like growth factor system: response to feed deprivation in lactating cows. Journal of Nutrition, 125, 493-502.

Molenaar, A.J., S.R. Davis & R.J. Wilkins (1992). Expression of alpha-lactalbumin, alpha-S1-casein, and lactoferrin genes is heterogeneous in sheep and cattle mammary tissue. Journal of Histochemistry and Cytochemistry, 40, 611-618.

Molenaar, A.J., Y.M. Kuys, S.R. Davis, R.J. Wilkins, P.E. Mead & J.W. Tweedie (1996). Elevation of lactoferrin gene expression in developing, ductal, resting, and regressing parenchymal epithelium of the ruminant mammary gland. Journal of Dairy Science, 79, 1198-1208.

Paape, M.J. & H.A. Tucker (1969). Influence of length of dry period on subsequent lactation in the rat. Journal of Dairy Science, 52, 518-522.

Phipps, R.H., D. Madakadze, T. Mutsvangwa, D.L. Hard & G. DeKerchove (1991). Use of bovine somatotropin in the tropics: the effect of sometribove on milk production of Bos indicus, dairy crossbred and Bos taurus cows in Zimbabwe. Journal of Agricultural Science, 117, 257-263.

Pitkow, H.S., R.P. Reece & G.L. Waszilycsak (1972). The integrity of mammary alveolar cells in two consecutive lactations. Proceedings of the Society for Experimental Biology and Medicine, 139, 845-850.

Quarrie, L.H., C.V.P. Addey & C.J. Wilde (1996). Programmed cell death during mammary tissue involution induced by weaning, litter removal, and milk stasis. Journal of Cellular Physiology, 168, 559-569.

Remond, B., A. Ollier & G. Miranda (1992). Milking of cows in late pregnancy: milk production during this period and during the succeeding lactation. Journal of Dairy Research, 59, 233-41.

Shayan, P., C. Gerlach, F.U. Hugel, G. Kay, J.D. Campbell, J. Gerdes & J.S. Ahmed (1999). The proliferation-associated nuclear protein Ki-67 in the bovine system: partial characterisation and its application for determination of the proliferation of Theileria-infected bovine cells. Parasitology Research, 85, 613-620.

Sinha, Y.N. & H.A. Tucker (1969). Mammary development and pituitary prolactin level of heifers from birth through puberty and during the estrous cycle. Journal of Dairy Science, 52, 507-512.

Smith, A., J.V. Wheelock & F.H. Dodd (1967). The effect of milking throughout pregnancy on milk secretion in the succeeding lactation. Journal of Dairy Research, 34, 145-150.

Smith, G.H. & D. Medina (1988). A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. Journal of Cell Science, 90, 173-184.

Sørensen, J.T. & C. Enevoldsen (1991). Effect of dry period length on milk production in subsequent lactation. Journal of Dairy Science, 74, 1277-1283.

Sørensen, M.T. & K. Sejrsen. (2003). Mammary Cell Proliferation, Apoptosis and Enzymatic Activity During Pregnancy and Lactation in Dairy Cows. In Proceedings of the 54th Annual Meeting of the European Association for Animal Production. Rome, Italy. Commission on Animal Physiology, Session Ph4.1 Free Communications.

Stelwagen, K., D.G. Grieve, B.W. McBride & J.D. Rehman (1992). Growth and subsequent lactation in primigravid Holstein heifers after prepartum bovine somatotropin treatment. Journal of Dairy Science, 75, 463-471.

Stelwagen, K., D.G. Grieve, J.S. Walton, J.L. Ball & B.W. McBride (1993). Effect of prepartum bovine somatotropin in primigravid ewes on mammogenesis, milk production, and hormone concentrations. Journal of Dairy Science, 76, 992-1001.

Swanson, E.W. (1965). Comparing continuous milking with sixty-day dry periods in successive lactations. Journal of Dairy Science, 48, 1205-1209.

Swanson, E.W., F.E. Pardue & D.B. Longmire (1967). Effect of gestation and dry period on deoxyribonucleic acid and alveolar characteristics of bovine mammary glands. Journal of Dairy Science, 50, 1288-1292.

Tsai, Y.C., Y. Lu, P.W. Nichols, G. Zlotnikov, P.A. Jones & H.S. Smith (1996). Contiguous patches of normal human mammary epithelium derived from a single stem cell: implications for breast carcinogenesis. Cancer Research, 56, 402-404.

Van Amburgh, M.E., D.M. Galton, D.E. Bauman & R.W. Everett (1997). Management and economics of extended calving intervals with use of bovine somatotropin. Livestock Production Science, 50, 15-28.

Vicini, J.L., F.C. Buonomo, J.J. Veenhuizen, M.A. Miller, D.R. Clemmons & R.J. Collier (1991). Nutrient balance and stage of lactation affect responses of insulin, insulin-like growth factors I and II, and insulin-like growth factor-binding protein 2 to somatotropin administration in dairy cows. Journal of Nutrition, 121, 1656-64.

Wilde, C.J., C.V.P. Addey, P. Li & D.G. Fernig (1997). Programmed cell death in bovine mammary tissue during lactation and involution. Experimental Physiology, 82, 943-953.

Wilde, C.J., C.H. Knight & D.J. Flint (1999). Control of milk secretion and apoptosis during mammary involution. Journal of Mammary Gland Biology and Neoplasia, 4, 129-136.

Milk fat depression: concepts, mechanisms and management applications

J.M. Griinari[1] and D.E. Bauman[2]
[1]University of Helsinki, Department of Animal Science, 00014 Helsinki, Finland
[2]Cornell University, Department of Animal Science, 14853, Ithaca, NY, USA

Abstract

Diet-induced milk fat depression (MFD) represents a model of extreme regulation of milk fat synthesis. MFD research conducted during the recent years has provided insight into specific milk fat inhibitors as well as potential mechanisms. To date, *trans*-10, *cis*-12 conjugated linoleic acid (10,12 CLA), produced as an intermediate in rumen biohydrogenation, is the only fatty acid shown unequivocally to reduce milk fat synthesis. Rumen digesta and milk fat of cows fed MFD diets have elevated concentrations of 10,12 CLA. However, the proportion of milk fat reduction attributable to 10,12 CLA formed in the rumen is often less than 50%, indicating a role for other unidentified inhibitors. Nevertheless, this has allowed for the development of novel tools for milk fat reduction with the target use in reducing energy requirement of early lactation cows. Milk fat production is a major energetic investment for the cow and milk fat reduction during a period of greatest energy need has the potential to influence productive functions. Multiple factors including nutritional status of the cow are likely to influence the partitioning of the energy spared from milk fat synthesis, and in some cases beneficial influences on tissue mobilization, milk yield and reproductive performance have been observed in response to the use of rumen-protected CLA supplements.

Keywords: milk fat depression, lactation, metabolism, conjugated linoleic acid

Introduction

Fat is the milk component with the highest energy content and its production constitutes a major energetic investment for the cow. Milk fat is also a component with a decreasing value in many markets. Thus, the investment in milk fat production in some cases provides marginal returns. Furthermore, milk fat is the most variable component in milk and its concentration and composition can be altered by nutrition of the dairy cow.

Milk fat depression represents a model of extreme regulation of milk fat synthesis. The biohydrogenation theory of milk fat depression has been recently proposed (Bauman and Griinari, 2001) and it provides a framework to understand and explain most, if not all, cases of diet-induced milk fat depression (MFD). Understanding the mechanism of MFD allows for regulation of milk fat production, mitigation of MFD when it is undesirable and inducing a reduction in milk fat secretion when it's economically advantageous.

Nutritional management of cows in early lactation targets specific requirements of the animal to maximize milk yield potential without compromising animal health and well-being and to

support reproductive functions. The first weeks postpartum are characterized by a rapid increase in milk secretion and the nutrient requirements for milk synthesis. However, the increase in feed intake is more gradual and this typically leads to a period of negative energy balance (Bauman and Elliot, 1983; Bell, 1995; Grummer, 1995). During the transition from pregnancy to lactation dairy cows go through an extensive series of metabolic adaptations over a relatively short period. These adaptations involve most if not all of the body tissues and are driven by the increased nutrient demand to support lactation (Bauman and Elliot, 1983; Bell, 1995). Extreme negative energy balance and nutritional imbalances predispose the cow to the occurrence of metabolic diseases and health problems that can impact milk production and profitability of the cow throughout the entire lactation (Gröhn *et al*., 1995; Drackley, 1999). Prolonged negative energy balance can also compromise reproductive performance (Butler, 2001).

A decrease in milk fat yield reduces the energy demand and thus has the potential to provide the cow in early lactation with more latitude to adapt and accommodate metabolic demands. Application of this concept currently drives development of novel tools for milk fat reduction. The following sections will provide a brief review on milk fat depression theory as well as on function of specific milk fat inhibitors and their use in the nutritional management of dairy cows in early lactation.

Milk fat depression

Diet-induced MFD has been extensively investigated over the past four decades and many theories have been advanced to explain its basis. The classic reviews of the first decade of research on diet-induced MFD focused on the potential role of substrate supply as the basis for limiting milk fat synthesis (Van Soest, 1963; Davis and Brown, 1970). Key reviews of 1980s emphasized the multi factorial nature of MFD while attributing a significant role to glucogenic-insulin theory (Bell, 1980; Engvall, 1980; Emery, 1988). The unique changes in *trans* fatty acids during diet-induced MFD was first recognized by Davis and Brown (1970) and was subsequently examined by several groups between 1976 and 1998 (see Bauman and Griinari, 2001 for references). This period of MFD research contributed to the concept that *trans* fatty acids were the main factor causing MFD (Selner and Schultz, 1980; Hagemeister, 1990; Erdman, 1996, 1999; Griinari *et al*., 1997). Recent reviews on milk fat depression (Bauman and Griinari, 2001; 2003; Griinari and Bauman, 2003) provide more details on the historic background and a more complete summary of past theories of MFD. In this paper we will only briefly visit the key features of diet-induced MFD and the main theories that have been advanced to explain MFD. The second part of this section discusses the biohydrogenation theory as a possible unifying theory.

Background

Fat is the most variable component of milk and diet-induced MFD is the most striking example of this variation. Historic review of the literature reveals that some of the diets still observed today to cause MFD were first described over 100 years ago. The oldest examples in the published literature were cited by Van Soest (1994) and Opstvedt (1984) and these involved feeding beets (reported by Boussingault in 1845) or supplements of fish oil (reported by Sebelien in 1894). Other conditions resulting in MFD include diets low in roughage and high in concentrates, diets

containing plant oils and diets based on forages that have reduced particle size (see Bauman and Griinari [2001] for references). Van Soest (1994) gives two additional examples: supplementation with anti-methanogenic substances and grazing pearl millet pastures. He concluded that the common effect of diets that reduce milk fat production was the reduction of methane and consequent elevation of propionate production in the rumen.

Increased propionate production in the rumen results in increased rates of gluconeogenesis in the liver and pancreatic release of insulin (Sutton, 1985). An increase in insulin secretion was suggested to reduce the release of free fatty acids from adipose tissue thereby reducing lipid precursor supply to the mammary gland (McClymont and Vallance, 1962). After it was discovered that insulin also stimulated glucose uptake and the use of acetate and butyrate for lipid synthesis in adipose tissue, the explanation to explain MFD was further extended to include a shortage of these lipogenic precursors and this has become known as the glucogenic-insulin theory (Van Soest, 1963; Ørskov *et al*., 1969; Jenny *et al*., 1974; Annison, 1976). The glucogenic-insulin theory will be included in the present review only as a classic example of a MFD theory based on reduced supply of lipogenic substrates.

Historically, MFD diets have been divided into two groups: one group consisting of diets that provide large amounts of readily digestible carbohydrates and reduced amounts of fibre, and a second group consisting of diets supplemented with oils high in polyunsaturated fatty acids (PUFA; e.g. plant and marine oils) (Davis and Brown, 1970). Today we know that these two groups are not independent in terms of inducing MFD. The presence of C18-PUFA is a prerequisite for MFD to occur when high grain/low fibre diets are fed (Griinari *et al*., 1998) and plant oil supplements will not depress milk fat yield if roughage intake is high or the effectiveness of roughage fibre is sufficient to maintain normal rumen function (Brown *et al*., 1962; Kalscheur *et al*., 1997). Thus, based on our current understanding of ruminal mechanisms involved in diet-induced milk fat depression, two dietary conditions are required for MFD to occur: (1) an alteration in rumen microbial processes and (2) the presence of sufficient amount of 18-carbon PUFA in the diet. Alterations in rumen microbial processes can be produced by high grain/low fibre diets, feeding forage with reduced particle size, the use of marine oil supplements and when anti-methanogenic compounds are added to the feed. Pearl millet pastures also appear to fall into this category based on the observed reduction in methane and increase in propionic acid production in the rumen (Van Soest, 1994). As indicated above, the presence of dietary 18-carbon PUFA is also a prerequisite and this suggests that diet-induced MFD involves ruminal metabolism of these unsaturated fatty acids. It is apparent that the amount of 18-carbon PUFA present in most feeds is sufficient to allow for formation of factors that result in MFD based on studies where MFD was induced by feeding high levels of corn grain without any dietary oil additions (Baldwin *et al*., 1969; Sutton *et al*., 1988; Palmquist and Schanbacher, 1991).

The theory, which is consistent with the formation of inhibitors of milk fat synthesis from 18-carbon precursors in the rumen involves *trans* fatty acids. *Trans* fatty acids are formed as intermediates in biohydrogenation of unsaturated fatty acids released during ruminal digestion of feeds. The mixture of *trans* fatty acids formed in the rumen is highly complex (Harfoot and Hazlewood, 1988; Griinari and Bauman, 1999). A simple description of the main biohydrogenation pathways of linoleic acid is presented in Figure 1. Davis and Brown (1970) were the first to recognize the

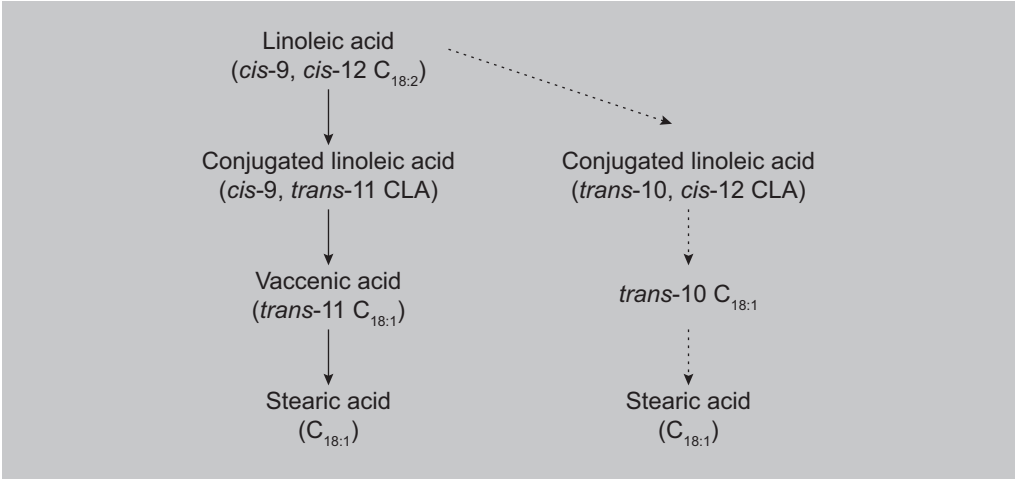

Figure 1. Pathways of rumen biohydrogenation of linoleic acid. Adapted from Griinari and Bauman (1999). The dashed line describes the shift that coincides with diet-induced MFD. In conditions of normal milk fat this represents a minor portion of the total biohydrogenation pathways.

relationship between *trans* 18:1 fatty acids and MFD and suggested their possible involvement in the mechanism of MFD.

Investigators who subsequently elaborated the *trans* fatty acid theory proposed these fatty acids may inhibit milk fat synthesis and they demonstrated that increases in milk fat content of *trans* 18:1 occurred for a wide range of diets related to MFD (Pennington and Davis, 1975; Astrup *et al*., 1976; Selner and Schultz, 1980; Banks *et al*., 1984; Hagemeister, 1990; Wonsil *et al*., 1994; Gaynor *et al*., 1994). Although milk fat content of *trans* 18:1 was generally correlated with the depression in milk fat percent, a summary of published literature revealed several inconsistencies (see review by Bauman and Griinari, 2001). Studies by Selner and Schultz (1980) and Kalscheur *et al.* (1997) provide examples where dietary manipulations resulted in substantial increases in milk fat content of *trans* 18:1 without a corresponding reduction in milk fat yield.

Trans-11 18:1 is the major *trans* 18:1 fatty acid present in milk fat (Molkentin and Precht, 1995), and it was generally assumed that the increase occurring with MFD was this isomer. An important development was the discovery that MFD was associated with a specific increase in *trans*-10 18:1, rather than *trans* 18:1 isomers in general (Griinari *et al*., 1998). The pathway for the formation of *trans*-10 18:1 in rumen biohydrogenation of linoleic acid is presented in Figure 1. Subsequent studies have extended the above results and established that an increased milk fat content of *trans*-10 18:1 is typically observed for all diets that cause MFD (see review by Bauman and Griinari, 2003). Thus, we conclude that in addition to an increase in *trans* 18:1 fatty acids in milk fat, the *trans*-10 shift in the major pathways of rumen biohydrogenation (Figure 1) is characteristic of diets that cause MFD. Consistent with a product/precursor relationship, we observed that milk fat concentrations of *trans*-10 18:1 were linearly related to *trans*-10, *cis*-12 CLA ($R^2 = 0.70$) for cows fed a high concentrate/low fiber diet (Griinari *et al*., 1999). Based on

this, we postulated that *trans*-10 18:1 or related metabolites formed in the rumen could be the cause of MFD (Griinari *et al.*, 1997; 1998).

Insulin-glucogenic theory - shortage of lipogenic precursors

Van Soest (1994) emphasized that a theory that adequately explains MFD has to account for the fact that the primary causative factor is produced in the rumen, and that it both promotes adipose tissue lipogenesis and at the same time attenuates lipogenesis in the mammary gland. A strong argument supporting the role of insulin in MFD is its recognized effect on lipid metabolism in adipose tissue, inhibition of lipid mobilization and stimulation of lipid synthesis (Vernon and Sasaki, 1991; Bauman, 2000). Also, the fact that daily variations in the circulating concentration of insulin have no apparent effect on glucose utilization by the mammary gland has resulted in a view that mammary gland is 'insensitive' to diet-related variations in circulating insulin. Both *in vitro* (Bauman *et al.*, 1973) and *in vivo* studies (Hove, 1978a, b; Laarveld *et al.*, 1985) demonstrated that insulin had no acute effect on glucose uptake consistent with ruminant mammary epithelial cells having only GLUT1 transporters (Zhao *et al.*, 1996). Thus, it is conceivable that stimulation of insulin secretion may affect nutrient partitioning to non-mammary tissues thereby limiting the supply and pattern of milk fat precursors available to the mammary gland.

The role of glucogenic nutrient supply in regulating milk fat synthesis has been examined in a number of studies using intra-gastric infusions of propionate and glucose (Figure 2). Reduction in milk fat yield appears to be independent of dose of ruminally infused propionate and the average reduction is just little over 10% (17 experimental treatments). When glucose is infused post-ruminally the average reduction is 6% and the nadir in milk fat response is produced by glucose infusions between 0.75 to 1.5 kg/d (33 experimental treatments).

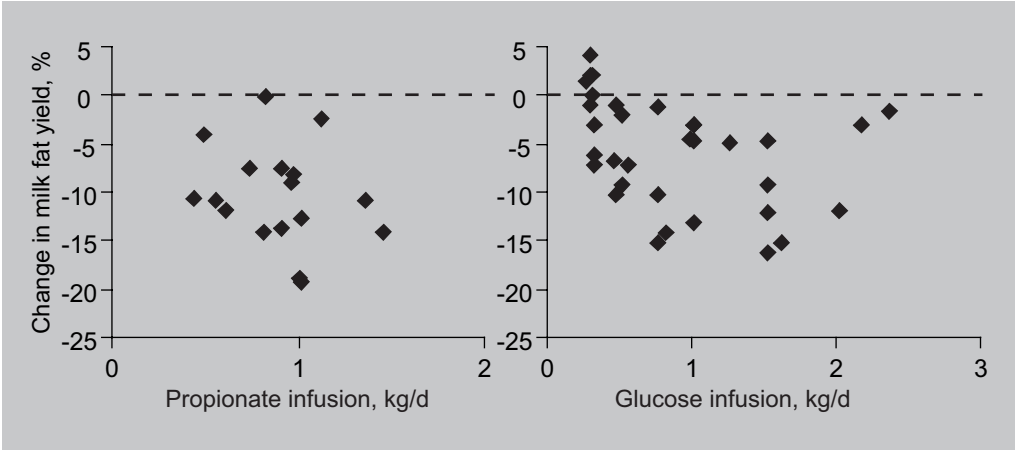

Figure 2. Summary of the effects of exogenous infusion of propionate (n = 17 experiments) or glucose (n = 33 experiments) on milk fat yield in dairy cows. Summaries are based on data previously reviewed in Bauman and Griinari (2001) and Griinari and Bauman (2003). Recent data added are propionate infusions by Rigout et al. *(2003) and glucose infusions by Hurtaud* et al. *(2000) and Rigout* et al. *(2002; 2003).*

The role of insulin on milk fat synthesis has also been investigated using the hyperinsulinemic-euglycemic clamp approach, which allows examination of the role of insulin without the complication of the hypoglycaemic effect of insulin (DeFronzo *et al*., 1979). Experiments were conducted with cows in mid-lactation (positive energy balance) and cows in early lactation (negative energy balance) (see Bauman and Griinari, 2003 for details). In early lactation, mobilized fat reserves represent a substantial source of precursors for milk fat and consistent with this, the anti-lipolytic effect of insulin reduced milk fat yield by 35%. The effect of insulin infusion in cows that were in positive energy balance, however, was minimal with the reduction in milk fat yield averaging 5%. This level of reduction is consistent with the proportion of milk fat fatty acids originating from body fat reserves in well-fed cows (Palmquist and Mattos, 1978; Pullen *et al*., 1989). In both situations this results in a reduction in the proportion of long chain fatty acids in milk fat, and this shift is exactly the opposite of what is observed in diet-induced MFD (Bauman and Griinari, 2000).

Overall, we conclude that observed reductions in milk fat synthesis in studies using intragastric infusions of insulin secretagogues (glucose or propionate) or intravenous infusions of insulin relate to insulin's well established role in inhibiting adipose tissue lipolysis and the magnitude of effects correspond to the proportion of milk fatty acids supplied by the tissue lipid reserves. In well-fed animals this source of fatty acids for milk fat synthesis is less than 10%. Furthermore, when the rate of milk fat synthesis and associated uptake of lipid precursors is reduced by direct effect of inhibitory compounds produced in the rumen, concurrent reduction in the supply of lipid precursors of smaller or equal magnitude will not result in net shortage in precursor supply. Although, inhibition of milk fat synthesis and shortage of lipid precursors can co-exist in the situation of diet-induced MFD, the contribution of reduced supply of lipid precursors, in the presence of inhibition, will be negligible. Thus, a single theory based on a direct inhibition of fat synthesis in the mammary gland by rumen derived factors would be sufficient to account for most, if not all, situations of diet-induced MFD.

Biohydrogenation theory

Recent investigations examining the transfer of CLA to milk fat in dairy cows produced the unexpected observation that CLA supplements infused post-ruminally resulted in a dramatic reduction in milk fat secretion (Loor and Herbein, 1998; Chouinard *et al*., 1999a; 1999b). Infused supplements consisted of a mixture of different isomers, one being *trans*-10, *cis*-12 CLA, the intermediate in the proposed pathways of biohydrogenation occurring under conditions of MFD (Figure 1). Baumgard *et al.* (2000) was the first to directly examine the role of *trans*-10, *cis*-12 CLA in MFD by utilizing post-ruminal infusions of pure CLA isomers. They demonstrated that *trans*-10, *cis*-12 CLA inhibited milk fat synthesis whereas the *cis*-9, *trans*-11 CLA isomer had no effect.

When low forage diets are fed to induce MFD, formation of *trans*-10, *cis*-12 CLA in the rumen is increased. Furthermore, the increase is associated with a reduction in milk fat synthesis and the effect of the diet on milk fat as well as on *trans*-10, *cis*-12 CLA production is alleviated by dietary inclusion of dietary buffers (Kalscheur *et al*., 1997; Piperova *et al*., 2000; Peterson *et al*., 2003). The relationship between milk fat percentage and *trans*-10, *cis*-12 CLA content in milk

fat is curvilinear (Griinari *et al*., 1999) further supporting the role of *trans*-10, *cis*-12 CLA as a milk fat inhibitor in diet-induced MFD. However, at comparable reductions in milk fat yield, the concentration range of *trans*-10, *cis*-12 CLA in milk fat from cows fed MFD diets is markedly less than the concentration in milk fat from studies where MFD was induced by post-ruminal infusions of pure *trans*-10, *cis*-12 CLA isomer (Figure 3). Studies with fish oil provide another example where the reduction in milk fat is not matched by increases in *trans*-10, *cis*-12 CLA (see review by Griinari and Bauman 2003). Recently, Loor *et al*. (2005) have shown that t10, c12 increase minimally in MFD cows when low forage diet is supplemented with linseed oil. These data suggest that in addition to *trans*-10, *cis*-12 CLA, other inhibitors of milk fat synthesis are formed in the rumen when MFD diets are fed (see reviews by Bauman and Griinari, 2001; 2003). Levels of *trans*-10, *cis*-12 CLA in milk fat from cows supplemented with Ca-salts of CLA are also lower than the levels observed in post-ruminal infusion studies at comparable reductions in milk fat yield (Figure 3). More recent studies involving diet-induced MFD or dietary supplements of CLA provide further evidence that there are additional bioactive fatty acids produced as intermediates in rumen biohydrogenation that regulate milk fat synthesis (Castañeda-Gutierrèz *et al*., 2005; de Veth *et al*., 2005; Loor *et al*., 2005).

To accommodate for the role of *trans*-10, *cis*-12 CLA and other potential inhibitors formed in the rumen, Bauman and Griinari (2001) proposed the "biohydrogenation theory" of MFD based on

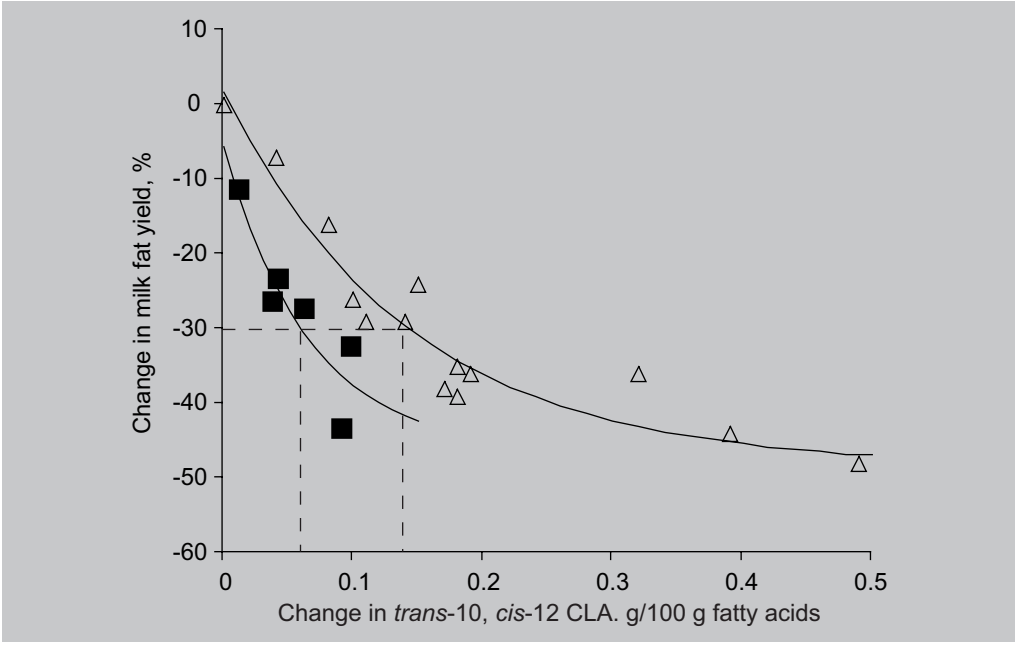

Figure 3. Relationship between milk fat yield and milk fat content of trans-10, cis-12 CLA during abomasal infusion of trans-10, cis-12 CLA (summary of seven studies; de Veth et al.*, 2004) (white symbols) or due to feeding of MFD diets (Piperova* et al.*, 2000; Peterson* et al.*, 2003) or feeding of Ca-salts of CLA (Giesy* et al.*, 2002; Perfield* et al.*, 2002) (black symbols).*

the concept that under certain dietary conditions the pathways of rumen biohydrogenation are altered to produce unique fatty acid intermediates, some of which are potent inhibitors of milk fat synthesis. This theory is consistent with the general requirements to achieve diet-induced MFD characterized earlier: 1) an alteration in rumen microbial processes and 2) the presence of sufficient amount of 18-carbon PUFA in the diet.

Trans-10, *cis*-12 CLA is the only fatty acid shown unequivocally to inhibit milk fat synthesis. To date, all the four CLA isomers present in the mixtures used in the initial CLA infusion studies (Loor and Herbein, 1998; Chouinard *et al*., 1999a; 1999b) including *trans*-8, *cis*-10, *cis*-9, *trans*-11 and *cis*-11, *trans*-13 CLA have been tested and only *trans*-10, *cis*-12 CLA has an effect (Baumgard *et al*., 2000; 2002a; Loor *et al*., 2002; Loor and Herbein, 2003; Perfield *et al*., 2004c). Likewise, *trans*-10, *trans*-12 CLA has been shown to have no effect on rates of milk fat synthesis (Perfield *et al*., 2004b). The original *trans* fatty acid theory of MFD assumed that *trans* 18:1 fatty acids were inhibitors of milk fat synthesis, but no specific *trans* 18:1 fatty acid has been rigorously tested so far. The increase in milk fat content of *trans*-10 18:1 and MFD reported in initial studies (e.g.Griinari *et al*., 1998; Newbold *et al*., 1998; Offer *et al*., 1999; Piperova *et al*., 2000; Offer *et al*., 2001) has been consistently observed (see summary of 13 studies by Loor *et al*., 2005) and this together with the product-precursor relationship between *trans*-10, *cis*-12 CLA and *trans*-10 18:1 (Griinari and Bauman, 1999) make *trans*-10 18:1 an obvious candidate. However, the temporal pattern of *trans*-10 18:1 enrichment in milk fat and the reduction in milk fat percentage observed with diets containing fish meal as a source of marine lipids do not correspond closely (AbuGhazaleh *et al*., 2004). This observation suggests that the *trans*-10 shift in the major biohydrogenation pathways may be a characteristic event associated with the shift in rumen biohydrogenation pathways and the resulting formation of specific milk fat inhibitors rather than this *trans* 18:1 isomer having a direct inhibitory effect. Also, the ratio between *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA content in milk fat (0.013 and 0.01; Griinari *et al*., 1999 and Piperova *et al*., 2002, respectively) and rumen digesta (0.008; Piperova *et al*., 2002) suggests that if *trans*-10 C18:1 did effect milk fat synthesis, its potency would be substantially less than *trans*-10, *cis*-12 CLA.

The effect of *trans*-10, *cis*-12 CLA on milk fat synthesis

A number of post-ruminal infusion studies have been conducted using a wide range of *trans*-10, *cis*-12 CLA doses (1.25 to 14 g/d) (Baumgard *et al*., 2000; 2001; 2002a; 2002b; Peterson *et al*., 2002; Loor and Herbein, 2003; Perfield *et al*., 2004c; de Veth *et al*., 2004). Data from these studies can be examined in an attempt to understand the dynamics of CLA-induced milk fat reduction and to develop hypotheses regarding the mechanism of inhibition.

Milk fat yield and fatty acid composition

De Veth *et al.* (2004) has presented the most extensive summary of milk fat responses involving post-ruminal infusion of *trans*-10, *cis*-12 CLA and used exponential decay models to describe the dose-response relationships (e.g. Figure 4). In this summary of seven studies, the maximum reduction in milk fat yield was about 50% of controls and the effective dose (abomasal infusion) to achieve a reduction of one-half of the maximum was approximately 2.6 g/d of *trans*-10, *cis*-12 CLA. Overall, the magnitude of the milk fat reduction achieved with abomasal infusion of

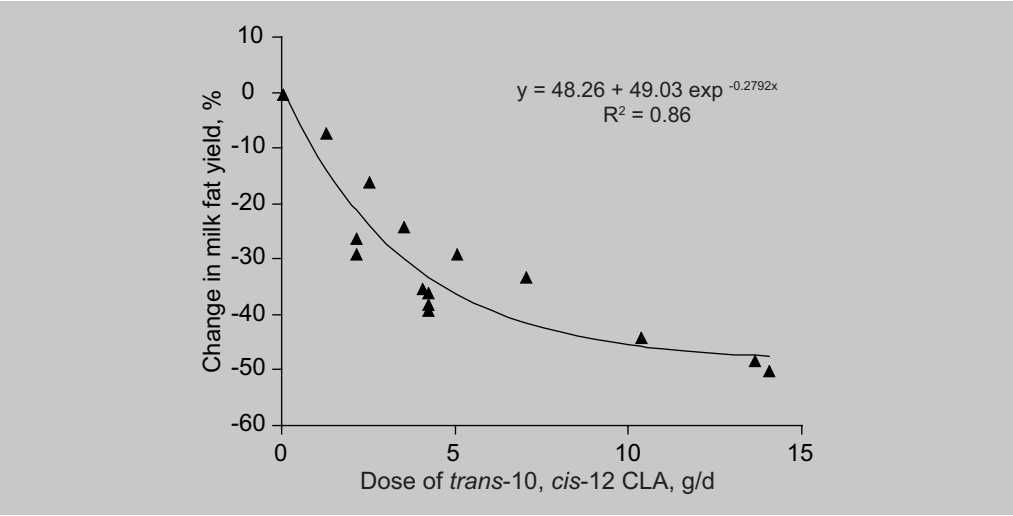

Figure 4. Relationship between milk fat yield and the dose of trans-10, cis-12 CLA abomasally infused in lactating cows (summary of seven studies as compiled by de Veth et al.*, 2004).*

trans-10, *cis*-12 CLA is similar to the maximum level of reduction observed in diet-induced MFD (Bauman and Griinari, 2001).

A significant decrease in milk fat percentage is observed within 24 h after the abomasal infusion of *trans*-10, *cis*-12 CLA begins and the temporal pattern is a progressive decrease over the first few days reaching nadir in about 4 to 5 days. Likewise, milk fat percentage returns to normal over a similar time interval when treatment is terminated (Baumgard *et al*., 2000, 2001; de Veth *et al*., 2004). Thus, the process whereby milk fat synthesis is inhibited occurs within hours and rescue from the inhibition is equally rapid.

Again, the exponential decay model developed by de Veth *et al*., (2004) is appropriate from a biological basis as a small increase in milk fat content of *trans*-10, *cis*-12 CLA below 0.2% of total fatty acids is associated with a relatively large decrease in milk fat yield and the nadir in the reduction of milk fat yield corresponds to a broad range of *trans*-10, *cis*-12 CLA concentrations with very little additional decrease in milk fat reduction when the *trans*-10, *cis*-12 CLA content exceeds 0.3 to 0.4% of total fatty acids.

Secretion of *trans*-10, *cis*-12 CLA in milk fat (g/d) is linearly related to the dose of this isomer (g/d) (see summary by de Veth *et al*., 2004). Further, the linear relationship indicates a relatively constant transfer efficiency (proportion of abomasally infused fatty acid secreted in milk fat) of 21.8% over a range of *trans*-10, *cis*-12 CLA doses (0 to 10 g/d) and it appears to be independent of the basal yields of milk and milk fat. These data summarized by de Veth *et al.* (2004) provide a basis for comparison between post-ruminal infusion and various protected formulations of CLA where 21.8% percent transfer efficiency represents 100% rumen by-pass and complete absorption in the intestine; transfer efficiencies less than this would reflect less complete protection from rumen

biohydrogenation and/or reduced intestinal bioavailability. The use of rumen protected CLA (RP-CLA) supplements results in an increase in the milk fat content of *trans*-10, *cis*-12 from ~0.01 to < 0.04% of total fatty acids (Perfield *et al*., 2002; Bernal-Santos *et al*., 2003; Castañeda-Gutièrrez *et al*., 2005). Various biological effects, both beneficial and less desirable have been reported in humans consuming CLA supplements (Kelley and Erickson, 2003), but the level of *trans*-10, *cis*-12 supplied by dairy products even cow's receiving RP-CLA supplements is much too low to be of biological significance. Studies infusing mixtures of CLA to lactating cows (Chouinard *et al*., 1999a, b; Perfield *et al*., 2004a) found that the transfer efficiency of *trans*-10, *cis*-12 CLA to milk fat was generally less than the transfer efficiency of the other CLA isomers (*trans*-8, *cis*-10; *cis*-11, *trans*-13 and *cis*-9, *trans*-11 CLA). Similar results have been observed when pure CLA isomers were abomasally infused in dairy cows (Baumgard *et al*., 2000; Loor and Herbein, 2003; Perfield *et al*., 2004c) It is unknown whether this lower transfer efficiency to milk fat is related to differences among isomers in use by non-mammary tissues, or due to differences in uptake and use by the mammary gland. Loor and Herbein (2003) infused post-ruminally 15 g/d either *cis*-9, *trans*-11 CLA or *trans*-10, *cis*-12 CLA and determined mammary extraction ratios based on enrichments of these isomers in plasma triglycerides and free fatty acids. They found that the extraction ratios were relatively similar between the two isomers thus providing no support for reduced uptake of *trans*-10, *cis*-12 CLA as an explanation for reduced transfer efficiency. Interestingly, the transfer of *trans*-10, *cis*-12 CLA to body fat in growing animals (rodents and pigs) and in tissue lipids as well as egg yolk lipids in laying hen has been shown to be much lower than the transfer of other CLA isomers (Kramer *et al*., 1998; de Deckere *et al*., 1999; Park *et al*., 1999; Schäfer *et al*., 2001; Ostrowska *et al*., 2003).

Earlier studies involving post-ruminal infusion of high doses of mixed CLA isomers or pure *trans*-10, *cis*-12 CLA (Chouinard *et al*., 1999a, b; Baumgard *et al*., 2000) observed a decreased secretion of all chain lengths of milk fatty acids, but the greatest decline occurred in short and medium chain fatty acids. These fatty acids mainly arise from de novo synthesis in the mammary gland and thus it was concluded that the inhibitory effect of CLA involved primarily de novo synthesis of fatty acids. This observation was consistent with earlier observations involving diet-induced MFD where a more dramatic reduction in de novo synthesized fatty acids occurred when a pronounced MFD was induced (Davis and Brown, 1970; Grummer, 1991; Palmquist *et al*., 1993; Erdman, 1999; Bauman and Griinari, 2000). Also at high doses of *trans*-10, *cis*-12 CLA the ratios of myristoleic to myristic (14:1/14:0), palmitoleic to palmitic (16:1/16:0) and oleic to stearic acid (18:1/18:0) in milk fat are altered (Chouinard *et al*., 1999a,b; Baumgard *et al*., 2000). These ratios, referred to as desaturase index, have been used as a proxy for Δ-9 desaturase activity and the consistent decreases observed at high CLA doses indicate a reduced activity or amount of Δ-9 desaturase. Subsequent infusion studies at doses of *trans*-10, *cis*-12 CLA that result in a less dramatic reduction in milk fat yield demonstrated, however, that the proportional reduction in milk fat fatty acids (molar basis) was similar for the fatty acids derived from de novo synthesis (< 16 carbons) and those that arise from the uptake of preformed fatty acids (> 16 carbons) (Baumgard *et al*., 2001; Peterson *et al*., 2002). Likewise, at these lower doses there was little or no effect on the fatty acid pairs representing substrate-product for Δ-9 desaturase (Baumgard *et al*., 2001; Peterson *et al*., 2002). Overall, these results indicate that the reduction in milk fat as a result of treatment with *trans*-10, *cis*-12 CLA is not dependent on a specific pronounced effect

on de novo synthesis or desaturation processes; rather effects must involve a coordinated down-regulation of the many, perhaps all, of the processes associated with the synthesis of milk fat.

Post-ruminal infusion studies have generally involved treatment periods of 4 to 5 d. Therefore, it is of interest to examine the effect of long-term milk fat reduction on fatty acid composition. Perfield *et al*. (2002) fed rumen protected CLA (4 isomer mixture) for 20 wk and induced an average 23% reduction in milk fat. In this long-term study, the decrease in the proportion of fatty acids derived from de novo synthesis (< 16 carbons), uptake of preformed fatty acids (> 16 carbons), and both sources (16 carbons) were constant across the 20 wk treatment period and similar among the different sources, although the proportion of fatty acids synthesized de novo was reduced to a slightly greater extent.

Mechanism of milk fat depression

The constancy of the reduction in de novo and preformed fatty acids after short term infusion (5 d) of low doses of *trans*-10, *cis*-12 CLA (< 5 g/d) suggests that the mechanism through which *trans*-10, *cis*-12 CLA inhibits milk fat synthesis involves coordinated regulation of key lipogenic enzymes. Baumgard *et al.* (2002b) demonstrated exactly this by using mammary tissue from cows infused post-ruminally with *trans*-10, *cis*-12 CLA (13.6 g/d). Reductions in mRNA abundance were observed for lipogenic genes involved in the uptake (lipoprotein lipase, LPL) and transport of fatty acids (fatty acid binding protein, FABP), de novo synthesis of fatty acids (acetyl CoA carboxylase, ACC; fatty acids synthase, FAS), desaturation (stearoyl-CoA desaturase, SCD) and triglyceride synthesis (acylglycerol phosphate acyl transferase, AGPAT; glycerol phosphate acyl transferase, GPAT). Furthermore, these reductions in mRNA abundance were similar in magnitude to the degree of reduction in milk fat yield.

The effect of diet-induced MFD on the mRNA abundance for mammary enzymes involved in milk fat synthesis has been examined by Piperova *et al.* (2000), Ahnadi *et al.* (2002) and Peterson *et al.* (2003). All showed similar results for common enzymes. The study by Peterson *et al.* (2003) was the most extensive evaluation and demonstrated clearly that the mechanism of diet-induced MFD involves a coordinated suppression of key lipogenic enzymes in the mammary gland. MFD was also characterized in this study by the appearance of *trans*-10, *cis*-12 CLA in the milk fat at a level that corresponded with milk fat reduction observed in other studies (see Figure 3). Levels of *trans*-10, *cis*-12 CLA in milk fat were found to be closely correlated with the reduction in transcript abundance for ACC and less closely, but still significantly with FAS, LPL and GPAT. Thus, the presence of *trans*-10, *cis*-12 CLA in milk fat is an important predictor of reductions in the uptake of circulating fatty acids, de novo fatty acid synthesis and triglyceride synthesis in the mammary gland in diet-induced MFD. This is significant considering that *trans*-10, *cis*-12 CLA appears to account for only up to 50% of the milk fat reduction when MFD diets or Ca-salts of CLA are fed (Figure 3). Therefore, it must be that the other inhibitors of milk fat synthesis, as yet unidentified, are closely associated with the altered pathways of rumen biohydrogenation that result in the formation of *trans*-10, *cis*-12 CLA.

Coordinated suppression of lipogenic genes in pathways of milk fat synthesis suggests that a central regulator of lipid synthesis is involved in MFD and the role of the sterol response element-

binding protein (SREBP) family of transcription factors was postulated (Peterson *et al*., 2003; Bauman and Griinari, 2003). The role of SREBP-1 in the regulation of lipid metabolism has been extensively characterized in rodents (Horton *et al*., 2002; McPherson and Gauthier, 2004; Pegorier *et al*., 2004). Peterson *et al.* (2004) were the first to examine different elements of the SREBP-regulatory system in a bovine mammary epithelial cell line (MAC-T) under conditions where *trans*-10, *cis*-12 CLA induced a reduction in fat synthesis. They elegantly demonstrated that the reduction in lipid synthesis corresponded to a decrease in mRNA abundance for ACC, FAS and SCD and in the abundance of the activated nuclear fragment of the SREBP-1 protein. Inhibition of the proteolytic activation of SREBP-1 and a subsequent reduction in migration of the active fragment to the nucleus by *trans*-10, *cis*-12 CLA is likely to reduce transcriptional activation of lipogenic genes consistent with the suppression of SREBP-1 activity by unsaturated fatty acids (see reviews by Jump, 2004; McPherson and Gauthier, 2004).

MFD is a mechanism that involves coordinated suppression of milk fat synthesis as demonstrated by the array of lipogenic enzymes that are altered. The established role of SREBP-1 in mammary cells appears to tie these coordinated responses together under one point of regulation. However, recent experimental results with model systems have shown a great diversity of fatty acid-sensor proteins and the list of potential transcription factors involved in fatty acid-mediated gene expression continues to grow (see review by Pegorier *et al*., 2004). Results to date demonstrate a role for SREBP in mediating the coordinated reduction in mammary lipogenic genes in response to *trans*-10, *cis*-12 CLA. Exploring other potential dimensions of this unique regulatory system awaits future studies.

Peterson *et al.* (2003) pointed out that if one pathway involved in lipid synthesis is acutely affected in such a manner (e.g. de novo synthesis), other pathways are often up regulated to compensate (e.g. uptake and transport of preformed fatty acids from circulation). There is compelling evidence in the classic literature on that effect. Virtanen (1966) fed lactating dairy cows a synthetic diet for the entire lactation, adding only minimal amounts of plant oil to the diet to prevent an essential fatty acid deficiency (37 to 130 g/d). During the period of low level of oil addition, milk fat percentage was maintained by increased rates of de novo fatty acid synthesis. Milk fatty acids synthesized de novo including palmitic acid increased dramatically from 54% on the standard diet to 84% of the total fatty acids for the low oil diets. However, if the inhibition of milk fat synthesis included enzyme steps in the final stages of milk fat synthesis then compensatory changes in rates of fatty acid synthesis and incorporation of preformed fatty acids would not be possible. The final steps of milk fat synthesis would include triglyceride synthesis, packaging and intracellular transport of fat globules and secretion of milk fat.

The curvilinear relationships between the dose of *trans*-10, *cis*-12 CLA or concentration of *trans*-10, *cis*-12 CLA in milk fat and change in milk fat yield (Figures 3 and 4) indicate that the inhibition of milk fat synthesis can reach a maximum level after which incorporation of the inhibitor to the milk fat continues with no further reduction in the rate of milk fat synthesis. These relationships may indicate that the inhibition of milk fat synthesis by *trans*-10, *cis*-12 CLA demonstrates a classical dose-response curve with a saturation V_{max} whereas the kinetic curve for *trans*-10, *cis*-12 CLA incorporation is a linear function. Summary of post-ruminal infusion data supports the linearity of *trans*-10, *cis*-12 CLA incorporation to milk fat (deVeth *et al*., 2004).

Nutritional challenges of cows in early lactation

Advances in genetic selection and management during the past several decades have resulted in remarkable increases in the productivity of dairy cows. The overall increases in milk yield are reflected in increases in peak milk production, which cows generally achieve within six weeks post partum. The successful transition from pregnancy to the onset of lactation and the rapid increase in milk yield to achieve full productive potential is dependent on an extensive series of physiological adaptations that include many, perhaps most, body tissues and all nutrient classes (Bauman and Currie, 1980). This rapid increase in nutrient demands for milk synthesis occurs during a period of more gradual increase in feed intake and as a consequence a negative energy balance occurs during early lactation (Bauman and Elliot, 1983; Bell, 1995). Therefore, adaptations in nutrient use to support the rapidly increasing demands of lactation are critical during this period.

Nutrient utilization, mobilization of reserves and milk production are coordinated in a manner that allows most cows to reach a level of milk production commensurate to their productive potential in just a few weeks without compromising health and well-being. However, in some cases the cow's ability to maintain the balance between lactation demands and nutrient utilization is compromised and this can adversely affect well-being. A recent summary of commercial dairy farms in Minnesota indicates that 25% of cows that left the dairy herd were culled during the first 60 d post partum (Godden *et al.*, 2003). Extreme negative energy balance and nutritional imbalances predispose the cow to the occurrence of metabolic diseases and health problems that can impact milk production and profitability of the cow during the entire lactation (Gröhn *et al.*, 1995; Drackley, 1999). A prolonged negative energy balance can also adversely affect reproductive performance (Butler, 2001). In addition to the level of milk yield, animal health and reproductive performance are important economic issues and therefore, nutritional management strategies that improve the cow's well-being during the critical early lactation period are called for.

The transition period is characterized by extensive adaptations in the use of major organic nutrients and energy. Estimates of increased requirements for energy and amino acids as compared to the supply of nutrients indicate major deficiencies during the first few days of transition from gestation to lactation (Bell, 1995). The primary adaptation in lipid and protein metabolism to accommodate lactation needs is the mobilization of tissue reserves. In the case of protein, estimates are few, but depletion-repletion and body composition studies indicate that 20-25% of total body protein could be mobilized without compromising animal health and well-being (Botts *et al.*, 1979; review by Chilliard, 1999). Overall, the contribution of labile tissue protein to support the needs of the mammary gland for protein precursors and for the needs of hepatic gluconeogenesis up to peak lactation is very limited (Bauman and Elliot, 1983). The greatest demand to draw on labile body protein, however, may exist during the first week or two after parturition when the cow is in substantial negative nitrogen balance (Bell, 1995).

Demands of the mammary gland for glucose are met by a large increase in glucogenic activity in the liver. Liver gluconeogenesis supplies almost completely for the increased needs of the mammary gland (Reynolds *et al.*, 2003), thus putting the liver in the center of metabolic adaptations in support of lactation. During the transition period, propionate contributes 50 to

60% to net glucose release by the liver, the contribution of lactate ranges from 15 to 20%, and 2 to 4% of the net glucose output could be produced from glycerol released from tissue mobilization of NEFA (Reynolds *et al*., 2003). By difference, amino acids may account for 20 to 30% during the transition period (Overton and Waldron, 2004).

The most impressive example of the adaptations in nutrient use in early lactation is perhaps the extensive mobilization of NEFA from adipose tissue. Plasma NEFA concentrations are commonly used as a predictor of tissue mobilization based on the fact they are derived exclusively from the mobilization of body fat stores and the close correlation between plasma concentration and the rate of entry of NEFA (Bauman *et al*., 1988; Pullen *et al*., 1989; Dunshea *et al*., 1988). Bell (1995) estimated that 10.7 mol/d of NEFA were mobilized in lactating dairy cows on d 4 post partum based on mean plasma concentration of 770 mmol/l and use of the equation derived by Pullen *et al.* (1989). Predictions were based on a milk production of 29.6 kg/d and feed consumption of 14.6 kg/d DM. This amount of NEFA is equivalent to the mobilization of about 3.2 kg/d triglyceride, in agreement with body composition studies showing a fat mobilization of 15-70 kg during 6 weeks post-partum (review by Chilliard, 1999). Furthermore, Bell (1995) predicted that NEFA mobilized from adipose tissue could have accounted for as much as 40% of milk fatty acids on d 4 post partum. This value is consistent with the observed reduction in milk fat synthesis (35%) obtained in an experiment using hyperinsulinemic-euglycemic clamp in cows d 10 post partum (see data in Bauman and Griinari, 2003). In this experiment, circulating concentrations of insulin were elevated fourfold over basal levels by i.v. infusion of insulin and this resulted in a decline in circulating NEFA concentrations from 1021 to 330 μmol/L.

Plasma NEFA concentrations decrease from the highest level during the first week of lactation to approximately one-half this value by 4 wk post partum (Burhans and Bell, 1998; Castañeda-Cutiérrez *et al*., 2005). Given that mammary extraction of NEFA is linearly related to arterial concentration (Miller *et al*., 1991) and that milk fat yield remains relatively constant during the first weeks of lactation, this level of plasma NEFA (350 μmol/L) could still account for as much as 20% of milk fat fatty acids.

As illustrated above, extensive mobilization of NEFA from adipose tissue is a normal adaptive response in high producing dairy cows. Excessive mobilization of body fat reserves, however, may occur in some cows predisposing these cows to metabolic disease (Gröhn *et al*., 1995; Drackley, 1999). The accumulation of lipid in the liver as a consequence of excessive mobilization of NEFA has been associated with impaired metabolic function of the liver (see review by Overton and Waldron, 2004), thereby resulting in effects such as increased formation of ketone bodies, reduced gluconeogenic capacity (Piepenbrink *et al*., 2004), a reduction in the capacity of the liver to convert ammonia to urea (Strang *et al*., 1998) and decreased capacity of the liver to clear bacterial endotoxins (Andersen *et al*., 1996). Fat mobilization and most likely lipid accumulation in the liver occur in all cows (Overton and Waldron, 2004). It is uncertain, though, when the use of reserves or fat accumulation in the liver may exceed the cow's ability to adapt so that health and well-being are compromised. Therefore, nutritional management strategies to minimize this possibility have application for the entire herd.

Nutritional management practices to achieve high levels of DM intake during the early lactation period are obviously of major importance and this includes feeding practices during the pre partum period (Grummer, 1995). As DMI is limiting in early lactation, traditional approaches to improve energy status include partial substitution of forages with more energy dense concentrates and the use of fat supplements (Grummer, 1995). However, high amounts of concentrate may predispose the cows in early lactation to digestive disturbances and increase incidence of lameness and displaced abomasum (Coppock *et al*., 1972; Nordlund, 1995; Bergsten, 1995). Also, strategies involving use of fat supplements have generally failed to reduce NEFA mobilization, body weight loss or alleviate negative energy balance in early lactation as a consequence of a corresponding decrease in feed intake and increase in milk energy secretion (see reviews by Chilliard, 1993 and Grummer, 1995). To support the maintenance of healthy liver function, supplements such as glucogenic precursors, monensin, choline or specific fatty acids have been used as supplements (Overton and Waldron, 2004).

Synthesis of milk fat represents approximately 50% of net energy requirement for lactation. Therefore, a nutritional strategy leading to a specific reduction in milk fat synthesis could conserve energy and provide the cow more latitude in the required metabolic adjustments. A reduction in milk fat secretion may translate into an improvement in energy status and this has the potential to influence productive functions that are sensitive to energy status i.e. mobilization of tissue reserves, achieving productive potential (peak milk yield) and maintaining normal reproductive functions.

Milk fat reduction and associated lactation responses

Strategic reduction in milk fat involving the use of rumen-protected formulation of conjugated linoleic acid (RP-CLA) is a novel approach in nutritional management of early lactation cows (Bauman *et al*., 2001; Griinari and Bauman, 2003). A reduction in milk fat while simultaneously maintaining feed intake and yield of other milk components is one of the potential outcomes of dietary supplementation with RP-CLA. Simply using current NRC (2001) formula to estimate net energy requirement for milk production demonstrates a reduction of approximately 3.3 Mcal/d in net energy requirement for lactation occurs when milk fat yield is reduced by 20% (milk yield 40 kg/d and reduction in fat percentage from 4.4 to 3.5%). One of the predicted consequences of reducing energy requirement by decreasing milk fat production is a reduced need to mobilize body fat. In a cow that may be marginal in terms of maintaining healthy liver function, this could represent a critical improvement.

Tissue mobilization

The effect of CLA-induced milk fat reduction on tissue mobilization and the magnitude of negative energy balance in early lactation has been examined in recent studies using RP-CLA supplements (Ca-salts of CLA consisting of a mixture of four *cis*/*trans* isomers). Reductions in milk fat yield varied from 8 to 30% depending on the dose of RP-CLA (Bernal-Santos *et al*., 2003; Moore *et al*., 2004; Castañeda-Gutiérrez *et al*., 2005). All of these studies initiated the treatments during the pre partum period (10 to 21 d before expected calving) and continued feeding the supplements 3 to 20 wk post partum. Thus, these investigations captured the period of maximum

changes in negative energy balance and plasma NEFA levels (Bauman *et al.*, 2001), but there were no significant treatment effects on estimated energy balance or plasma NEFA between cows receiving CLA supplements and the non-treated controls. In the study by Moore *et al.* (2004), net energy balance tended to improve (less negative) at the highest levels of CLA supplement intake and milk fat reduction (-22 and –30%), but there was no dose-response effect. Days to negative energy balance nadir were decreased by CLA supplementation (7.3, 5.8, 4.8 and 2.6 for the lowest to highest dose of CLA). This response may be relevant in terms of reproductive performance as recovery from negative energy balance nadir in early lactation provides an important signal for initiating ovarian activity (Beam and Butler, 1999) and days to energy balance nadir is highly correlated with days to first ovulation (Butler, 2001). Minor increases in milk yield and decreases in feed intake observed in these studies may explain the minimal changes in energy balance estimates when the effects are aggregated with relatively small reductions in milk fat yield.

A lack of significant effects on net energy balance and tissue mobilization during CLA supplementation in early lactation, even though there were significant reductions in milk energy output, raises the possibility that energy expenditure or excretion of urinary or faecal energy may have increased. An increased rate of metabolism and associated heat production plus increased faecal energy secretion has been reported in growing mice supplemented with CLA (West *et al.*, 1998; Terpstra *et al.*, 2002), although the dose of *trans*-10, *cis*-12 CLA was over 10-fold greater on a metabolic body weight basis than used in studies with dairy cows (Bauman *et al.*, 2003). Shingfield *et al.* (2004) conducted the first energy balance study to examine this in lactating dairy cows using excreta collection and respiration calorimetry to determine the partitioning of dietary gross energy in milk production. A RP-CLA supplement (casein formaldehyde encapsulated product consisting of two major CLA isomers) was fed at a level to supply 14.3 g/d of *trans*-10, *cis*-12 CLA. Treatment began immediately after calving and a 35% decrease in milk fat yield was observed. Most importantly, it was found that losses in heat, methane and faeces were not different between CLA-fed animals and the controls, although proportions of gross energy excreted in urine and milk were reduced. As a result, there was an increase in proportion of gross energy retained in body tissues. Thus, in the study by Shingfield *et al.* (2004), energy spared from the reduction in milk fat yield was partitioned towards body tissues demonstrating that the energy spared by milk fat reduction has the potential to improve energy status in early lactation cows. Multiple factors including nutritional status are likely to influence the partitioning of the spared energy, and this is an area requiring further study.

Decreased milk fat synthesis and associated decreased uptake of NEFA by the mammary gland was initially hypothesized to result in corresponding reduction in rates of lipolysis in adipose tissue and consequently reduced NEFA levels. It is perplexing that the early lactation studies involving use of RP-CLA supplements (Bernal-Santos *et al.*, 2003; Moore *et al.*, 2004; Shingfield *et al.*, 2004; Castañeda-Gutiérrez *et al.*, 2005) did not observe any effect on plasma NEFA levels even though milk fat yield was reduced. As discussed above, plasma levels of NEFA closely correlate with fat mobilization from adipose tissue. Elevated levels of plasma NEFA in early lactation are due to adaptations in adipose tissue that include increased rates of lipolysis and a suppression of fatty acid re-esterification in adipose tissue (reviewed by Bell, 1995; Chilliard, 1999). It is conceivable that the decreased use of lipogenic precursors in the mammary gland during CLA-induced suppression of milk fat synthesis does not down regulate the lipolytic signals in adipose

tissue, but may result in increased uptake of NEFA by other tissues thus sparing the oxidation of other nutrients. These changes may occur without apparent change in plasma concentration of NEFA.

Milk production

Nutritional status plays an important role in modulating the expression of the animal's genetic potential and in establishing the peak milk yield in early lactation (Bauman and Elliot, 1983; Bauman *et al.*, 1985; Drackley, 1999). Nutrients are not only sources of energy and building blocks for tissue components, but also sources of important metabolic signals modulating the physiological controls that regulate productive processes. Energy status, as a component of the overall nutritional status, can be altered by strategic reduction in milk fat synthesis. The potential effects of this change in milk production will be discussed in this section.

Initially the theory was that early lactation would represent a physiological state most responsive to CLA-induced reduction in milk fat synthesis and consequent improvement in energy status (Griinari and Bauman, 2003). Consistent with this, observed increases in milk yield in response to RP-CLA supplementation occurred in studies conducted in early lactation and the effects on milk yield were minor in studies conducted in established lactation and involving short-term infusions of CLA (summarized in Griinari and Bauman, 2003). Improvement in energy status in early lactation may produce a signal of nutritional adequacy, which then stimulates the development of the productive capacity and results in increased peak milk yield. In contrast, during established lactation well-fed cows are already producing at their productive potential. Studies conducted at Cornell University (Perfield *et al.*, 2002; Bernal-Santos *et al.*, 2003; Castañeda-Gutiérrez *et al.*, 2005) provide a useful set of examples illustrating the variation in milk production responses to RP-CLA supplements. Perfield *et al.* (2002) and Bernal-Santos *et al.* (2003) represent companion experiments conducted at two different stages of lactation (mid to late lactation and early lactation, respectively) using the same quantity and type of RP-CLA. The study reported by Castañeda-Gutiérrez *et al.* (2005) was also conducted in early lactation and it used two levels of RP-CLA, the lower dose matching the *trans*-10, *cis*-12 CLA dose in the study by Bernal-Santos *et al.* (2003) and the higher dose at a 2X level.

Stage of lactation and the duration of CLA treatment appear to influence CLA-induced inhibition of milk fat content. CLA-supplementation during early lactation period demonstrated minimal reduction in milk fat content during the first weeks immediately after calving (Bernal-Santos *et al.*, 2003). The difference in milk fat percentage between control and CLA-supplemented cows increased gradually and this pattern continued until dry-off (Figure 5). The study by Moore *et al.* (2004) demonstrated that a reduction in milk fat content can be observed already during the first week of lactation and a near maximal decrease achieved after two weeks post partum (-50%) by increasing the dose of CLA.

Milk yield was increased in one study conducted in early lactation (Bernal-Santos *et al.*, 2003), but not in the companion study conducted in late lactation (Perfield *et al.*, 2002) consistent with the proposed importance of the physiological state. In contrast, Castañeda-Gutiérrez *et al.* (2005) study, which involved RP-CLA supplementation immediately post partum did not observe any

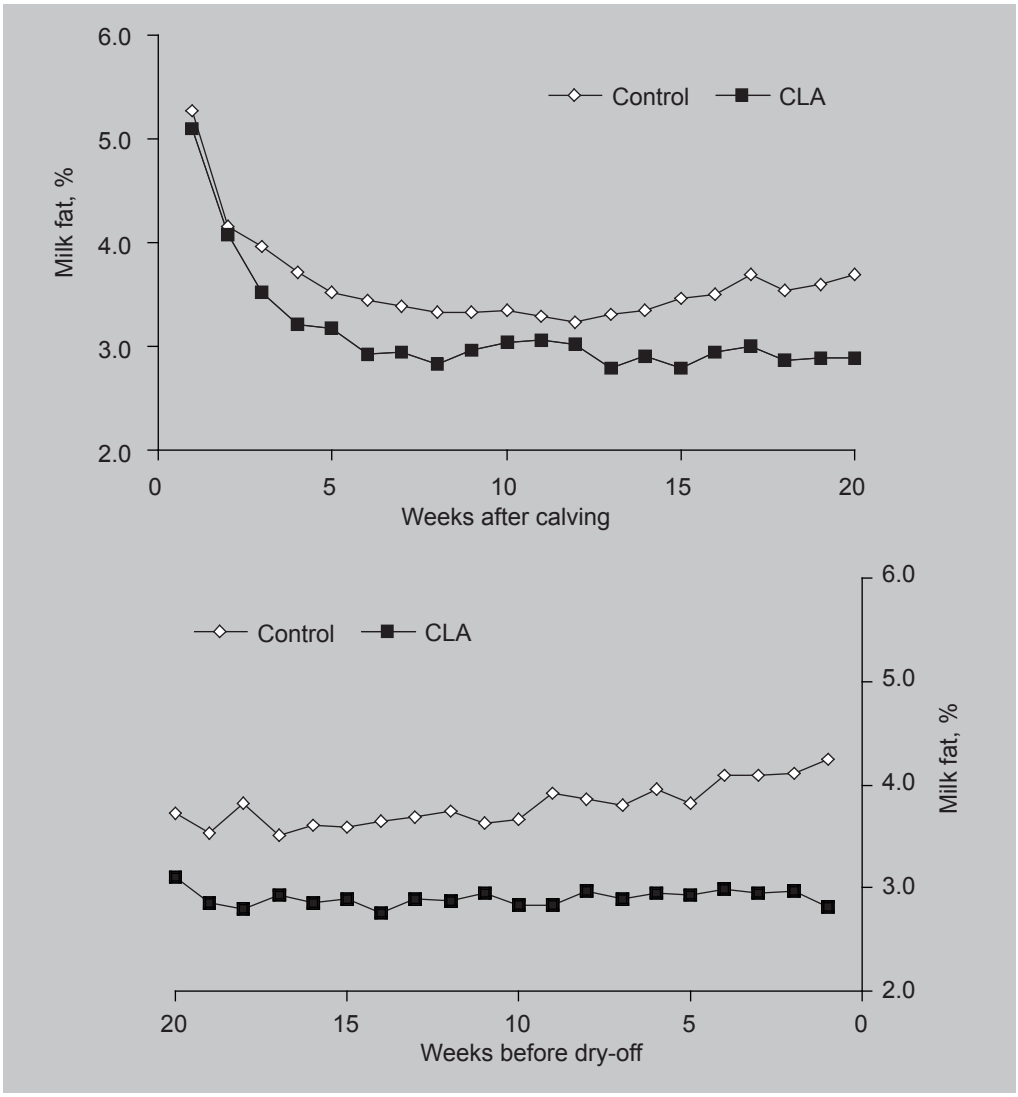

Figure 5. The effect of CLA-supplementation on milk fat percentage during the first and the last 20 wk of lactation (Source: adapted from Bernal-Santos et al. *(2003) and Perfield* et al. *(2002)).*

effect on milk yield. In order to examine the effect of nutritional status in explaining milk yield responses in the early lactation studies, metabolizable energy (ME) and metabolizable protein (MP) balance were determined by using the Cornell Net Carbohydrate and Protein System (Fox *et al.*, 2004). The diet in the study of Castañeda-Gutiérrez *et al.* (2005) provided a more adequate diet in terms of ME and MP requirements (see details in Castañeda-Gutiérrez *et al.*, 2005) suggesting that nutrient status may influence whether the CLA-induced reduction in milk fat secretion results in a corresponding increase in milk yield.

The studies by Bernal-Santos *et al.* (2003) and Castañeda-Gutiérrez *et al.* (2005) also examined effects of RP-CLA on milk protein composition and found no changes in proportion of casein, whey or NPN of total protein. In general, milk protein percentage is not influenced with the exception of one study involving RP-CLA supplementation on pasture. This study demonstrated a significant increase in protein percentage (Medeiros *et al*., 2000). Pasture studies involving short-term supplementation with RP-CLA in established lactation have observed increased milk yield and/or protein yield (Gulati *et al*., 2001; Mackle *et al*., 2003). Milk and protein yield responses in established lactation suggest that in the presence of energy limitation, a reduction in milk fat synthesis may allow for repartitioning of nutrients to allow increased milk and milk protein yields provided that amino acid supply is sufficient. These responses further suggest that the level and quality of protein supply may be important factors determining production responses when cows are supplemented with RP-CLA.

Reproductive performance

A strategy for reducing milk fat production in early lactation and its effects on reproduction in dairy cows is of special interest given established relationships between energy status and reproductive performance (Butler, 2001). Data of Bernal-Santos *et al.* (2003) clearly demonstrate that CLA had no adverse effects on the initial events in reproduction. The number of cows was too limited for proper evaluation of reproductive performance, but it is important to note that days to first oestrus and conception rate were numerically improved in this study, even though milk yield was increased. The study by Castañeda-Gutiérrez *et al.* (2005) is consistent with these data showing a tendency for improved reproductive performance when cows are supplemented with RP-CLA. It is intriguing that in the presence of clear tendencies towards improved reproductive performance, estimated net energy balance and plasma NEFA concentrations were unaffected (Bernal-Santos *et al*., 2003; Castañeda-Gutiérrez *et al*., 2005). These observations suggest that potential mechanisms independent of energy balance need to be considered.

The role of fat supplements in reproduction has been extensively investigated. Initially the effect of fat supplements was linked with the improved energy balance and associated more rapid return of ovulatory estrous cycles (Grummer and Carrol, 1991). Lucy *et al.* (1992) suggested it was the presence of specific fatty acids and not the additional energy provided that stimulated ovarian function. Fatty acids may influence reproduction through increased ovarian steroidogenesis, manipulation of insulin to stimulate ovarian follicle development, and/or inhibition of the uterine production and release of $PGF_{2\alpha}$ (Staples *et al*., 1998; Mattos *et al*., 2000).). In the bovine, maternal recognition of pregnancy is dependent on the secretion of interferon tau (IFNτ) by the developing embryo. IFNτ inhibits the synthesis of $PGF_{2\alpha}$ by the uterus preventing luteolysis (reviewed by Wolf *et al*., 2003). Thus, inhibition of $PGF_{2\alpha}$ synthesis by fatty acid supplementation can decrease embryonic loss when IFNτ secretion is not sufficient (see review by Abayasekara and Wathes, 1999). CLA has been reported to have an effect on prostaglandin synthesis in several different species and cell systems (see review by Belury, 2002). Of special interest is the inhibition of $PGF_{2\alpha}$ observed in uterine tissue of pregnant rats supplemented with CLA (Harris *et al*., 2001), and the decreased production of $PGF_{2\alpha}$ in endometrial cells incubated with CLA (Cheng *et al*., 2003).

The mechanism by which one or several CLA isomers could decrease $PGF_{2\alpha}$ production in tissues is not known, but a role for CLA metabolites (conjugated C18:3, C20:3) in decreasing tissue levels of arachidonic acid (C20:4) has been proposed (Banni, 2002). Arachidonic acid is a substrate for cyclooxygenase and lipoxygenase pathways of eicosanoid synthesis and it is conceivable that biosynthesis of eicosanoids would be affected by reduced availability of arachidonic acid. CLA-induced decrease of arachidonic acid has been observed in several animal models and cell cultures (reviewed by Banni, 2002). The effect of CLA on arachidonic acid levels is further influenced by the dietary supply of linoleic acid, precursor of arachidonic acid (Banni, 2002; Sébédio *et al*., 2003). Studies using rats fed a fat free diet or diets low in essential fatty acids (butter) have produced the highest levels of CLA metabolites in tissue lipids (Sébédio *et al*., 1997, 2001; Banni *et al*., 2001). Interestingly, lambs producing CLA naturally demonstrated extensive metabolism of CLA up to the conjugated C20:4 (Banni *et al*., 1996). This observation is consistent with ruminant animals having a limited supply of essential fatty acids (including linoleic acid) from their diet due to extensive rumen biohydrogenation. Perhaps this is a metabolic adaptation because ruminants appear to have a lower requirement for essential fatty acids in comparison to non-ruminants (Mattos and Palmquist, 1977).

Given that ruminant lipid metabolism functions in an essentially low linoleic acid state, it is possible that specific dietary fatty acids, supplied in sufficient amounts may interact with linoleic acid metabolism, synthesis of arachidonic acid and production of its metabolites. The reduction of uterine secretion of $PGF_{2\alpha}$ in cows fed fish oil has been demonstrated (Mattos *et al*., 2002; 2004) and this change was associated with an almost six fold increase in concentration of the main long chain polyunsaturated fatty acids, eicosapentaenoic (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6) in caruncular tissues. Interestingly, the ratio between EPA+DHA and linoleic acid in caruncles was increased dramatically form 0.07 to 0.51. In this study, fish oil feeding for 3 wk pre partum provided the cows 128 g/d of dietary EPA + DHA. It can be estimated that maximum 10% of those fatty acids escape rumen biohydrogenation intact and get absorbed (Doreau and Chilliard, 1997). Milk fat secretion of EPA + DHA increased by 6.4 g/d thus allowing the other half of absorbed n-3 fatty acids be metabolised and deposited in tissues. These numbers suggest that relatively small amounts of fatty acids delivered post-ruminally may become enriched in tissues lipids at levels that have the potential to modulate the metabolism of essential fatty acids and production of number of important lipid mediators such as prostaglandins and other eicosanoids.

Conclusions

Diet-induced milk fat depression occurs as a result of changes in the rumen function characterized by a shift in predominant pathways of biohydrogenation. The shift in rumen biohydrogenation associated with milk fat depression involves increased formation of *trans*-10 18:1 instead of *trans*-11 18:1 as an intermediate of linoleic acid biohydrogenation. *Trans*-10, *cis*-12 CLA, a precursor of *trans*-10 18:1 in the rumen, is currently the only biohydrogenation intermediate unequivocally shown to inhibit milk fat synthesis.

Studies involving post-ruminal infusions of *trans*-10, *cis*-12 CLA demonstrate the potency of this isomer and also provide data suggesting that *trans*-10, *cis*-12 CLA is not the only biohydrogenation

intermediate with the ability to inhibit milk fat synthesis. Identity of the other milk fat inhibitors formed in the rumen as intermediates of biohydrogenation is yet to be determined. Post-ruminal infusion studies also provide valuable information on the effect of the milk fat inhibitor on milk fat composition. Observed changes in milk fat composition suggest that the inhibition of milk fat synthesis involves coordinated regulation of key lipogenic enzymes. Mammary tissue mRNA abundance for all lipogenic genes studied indicates similar degree of reduction associated with diet-induced milk fat depression as well as reductions in milk fat synthesis induced by post-ruminal infusion of the milk fat inhibitor. Role of the sterol response element binding protein (SREBP) family of transcription factors as a central regulator of lipid synthesis has been indicated.

Reduction in milk fat reduces the energy requirement of milk production and has the potential to benefit productive functions of early lactation cows. Positive influences on tissue mobilization, milk yield and reproductive performance have been observed in cows supplemented with rumen protected CLA. The mechanism by which CLA influences the productive functions may involve the partitioning of energy spared from milk fat synthesis as well as modulation of essential fatty acid metabolism.

References

Abayasekara, D.R. & D.C. Wathes (1999). Effects of altering dietary fatty acid composition on prostaglandin synthesis and fertility. Prostaglandins, Leukotrienes & Essential Fatty Acids, 61, 275-287.

AbuGhazaleh, A.A., D.J. Schingoethe, A.R. Hippen & K.F. Kalscheur (2003). Conjugated linoleic acid and vaccenic acid in rumen, plasma, and milk of cows fed fish oil and fats differing in saturation of 18 carbon fatty acids. Journal of Dairy Science, 86, 3648-3660.

Ahnadi, C.E., N. Beswick, L. Delbecchi, J.J. Kennelly & P. Lacasse (2002). Addition of fish oil to diets for dairy cows. II Effects on milk fat and gene expression of mammary lipogenic enzymes. Journal of Dairy Research, 69, 521-531.

Andersen, P.H., N. Jarlov, M. Hesselholt & L. Baek (1996). Studies on in vivo endotoxin plasma disappearance times in cattle. Journal of Veterinary Medicine A, 43, 93–101.

Annison, E.F. (1976). Energy utilization in the body. In: H. Swan & W.H. Broster (eds.) Principles of Cattle Production. Butterworths, London, UK, 438pp.

Astrup, H.N., L. Vik-Mo, A. Ekern & F. Bakke (1976). Feeding protected and unprotected oils to dairy cows. Journal of Dairy Science, 59, 426-430.

Baldwin, R.L., H.J. Lin, W. Cheng, R. Cabrera & M. Ronning (1969). Enzyme and metabolite levels in mammary and abdominal adipose tissue of lactating dairy cows. Journal of Dairy Science, 52, 183-187.

Banks, W., J.L. Clapperton, A.K. Girdler & W. Steele (1984). Effect of inclusion of different forms of dietary fatty acid on the yield and composition of cow's milk. Journal of Dairy Research, 51, 387-395.

Banni, S. (2002). Conjugated linoleic acid metabolism. Current Opinion in Lipidology, 13, 261-266.

Banni S, G. Carta, E. Angioni, E. Murru, P. Scanu, M.P. Melis, D.E. Bauman, S.M. Fischer & C. Ip (2001). Distribution of conjugated linoleic acid and metabolites in different lipid fractions in the rat liver. Journal of Lipid Research, 42, 1056-1061.

Banni, S., G. Carta, M.S. Contini, E. Angioni, M. Deiana, M.A. Dessì, M.P. Melis & F.P. Corongiu. (1996). Characterization of conjugated diene fatty acids in milk, dairy products and lamb tissues. Journal of Nutritional Biochemistry, 7, 150-155.

Bauman D.E. (2000). Regulation of nutrient partitioning during lactation: homeostasis and homeorhesis revisited. In: P.B. Cronje (ed.) Ruminant Physiology: Digestion, Metabolism, Growth, and Reproduction., CAB International, New York, NY, 474pp.

Bauman, D.E., B.A. Corl, L.H. Baumgard & J.M. Griinari (2001). Conjugated linoleic acid (CLA) and the dairy cow. In: P.C. Garnsworthy & J. Wiseman (eds.) Recent Advances in Animal Nutrition. Nottingham University Press, Nottingham, UK, pp. 221-250.

Bauman, D.E., B.A. Corl & D.G. Peterson (2003). The biology of conjugated linoleic acid (CLA) and the dairy cow. In: J.L. Sébédio, W.W. Christie & R.O. Adlof (eds.) Advances in Conjugated Linoleic Acid Research, Vol. 2. AOCS Press, Champaign, IL, 337pp.

Bauman, D.E. & W.B. Currie (1980). Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. Journal of Dairy Science, 63, 1514-1529.

Bauman, D.E. & J.M. Elliot (1983). Control of nutrient partitioning in lactating ruminants. In: T.B Mepham (ed.) Biochemistry of Lactation. Elsevier Science Publishers B.V.: Amsterdam, The Netherlands, 500pp.

Bauman, D.E. & J.M. Griinari (2000). Regulation and nutritional manipulation of milk fat: low milk fat syndrome. In: J.A. Mol & R.A. Clegg (eds.) Biology of the Mammary Gland. Kluwer Academic/Plenum Publishers, New York, 314pp.

Bauman, D.E. & J.M. Griinari (2001). Regulation and nutritional manipulation of milk fat: Low-fat milk syndrome. Livestock Production Science, 70, 15-29.

Bauman, D.E. & J.M. Griinari (2003). Nutritional regulation of milk fat synthesis. Annual Review of Nutrition, 23, 203-227.

Bauman, D.E., D.L. Ingle, R.W. Mellenberger & C.L. Davis (1973). Factors affecting in vitro lipogenesis by bovine mammary tissue slices. Journal of Dairy Science 56,1520-1525.

Bauman, D.E., S.N. McCutcheon, W.D. Steinhour, P.J. Eppard & S.J. Sechen (1985). Sources of variation and prospects for improvement of productive efficiency in the dairy cow: A review. Journal of Animal Science, 60, 583-592.

Bauman, D.E., C.J. Peel, E.D. Steinhour, P.J. Reynolds, H.F. Tyrrell, A.C.G. Brown & G.L. Haaland (1988). Effect of bovine somatotropin on metabolism of lactating dairy cows: influence on rates of irreversible loss and oxidation of glucose and non-esterified fatty acids. Journal of Nutrition, 118, 1031-1040.

Baumgard, L.H, B.A. Corl, D.A. Dwyer & D.E. Bauman (2002a). Effects of conjugated linoleic acids (CLA) on tissue response to homeostatic signals and plasma variables associated with lipid metabolism in lactating dairy cows. Journal of Animal Science, 80, 1285-1293

Baumgard, L.H., B.A. Corl, D.A. Dwyer, A.Saebo & D.E. Bauman (2000). Identification of the conjugated linoleic acid isomer that inhibits milk fat synthesis. American Journal of Physiology, 278, R179-R184.

Baumgard, L.H., E. Matitashvili, B.A. Corl, D.A. Dwyer & D.E. Bauman 2002b. Trans-10, *cis*-12 conjugated linoleic acid decreases lipogenic rates and expression of genes involved in milk lipid synthesis in dairy cows. Journal of Dairy Science, 85, 2155-2163.

Baumgard, L.H., J.K. Sangster & D.E. Bauman (2001). Milk fat synthesis in dairy cows is progressively reduced by increasing supplemental amounts of trans-10, cis-12 conjugated linoleic acid (CLA). Journal of Nutrition, 131,1764-1769.

Beam, S.W. & W.R. Butler (1999). Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. Journal of Reproduction and Fertility, 54, 411-424.

Bell, A. W. (1980). Lipid metabolism in liver and selected tissues and in the whole body of ruminant animals. Progress in Lipid Research, 18, 117-164.

Bell, A.W. (1995). Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of Animal Science, 73, 2804-2818.

Belury, M.A. (2002). Inhibition of carcinogenesis by conjugated linoleic acid: Potential mechanisms of action. Journal of Nutrition, 132, 2995-2998.

Bergsten, C. (1995). Management and nutritional effects on foot health in dairy cows. In: EAAP Proceedings, Prague, Czech Republic.

Bernal-Santos, G., J. W. Perfield II, D. M. Barbano, D.E. Bauman & T. R. Overton (2003). Production responses of dairy cows to dietary supplementation with conjugated linoleic acid (CLA) during the transition period and early lactation. Journal of Dairy Science, 86, 3218-3228.

Botts, R.L., R.W. Hemken & L.S. Bull (1979). Protein reserves in the lactating dairy cow. Journal of Dairy Science, 62, 433-441.

Brown, W.H., J.W. Stull & G.H. Stott (1962). Fatty acid composition of milk. I. Effect of roughage and dietary fat. Journal of Dairy Science, 45, 191-196.

Burhans, W.S. & A.W. Bell (1998). Feeding the transition cow. In: Proceedings Cornell Nutrition Conference for Feed Manufacturers, Cornell University, Ithaca, NY. pp. 247–258.

Butler, W.R. (2001) Nutritional effects on resumption of ovarian cyclicity and conception rate in postpartum dairy cows. Animal Science, Occasional Publication, 26, 133-145.

Castañeda-Gutiérrez, E., T.R. Overton, W.R. Butler & D.E. Bauman (2005). Dietary supplements of two doses of calcium salts of conjugated linoleic acid during the transition period and early lactation. Journal of Dairy Science, 88, 1078-1089.

Cheng, Z., M. Elmes, D.R.E. Abayasekara & D.C. Wathes (2003). Effects of conjugated linoleic acid on prostaglandins produced by cells isolated from maternal intercotyledonary endometrium, fetal allantochorion and amnion in late pregnant ewes. Biochimica et Biophysica Acta, 1633, 170-178.

Chilliard Y., 1993. Dietary fat and adipose tissue metabolism in ruminants, pigs and rodents: a review. Journal of Dairy Science, 76, 3897-3927

Chilliard Y., 1999. Metabolic adaptations and nutrient partitioning in the lactating animal. In: J. Martinet, L.M. Houdebine and H.H. Head (Editors), Biology of Lactation. Collection Mieux Comprendre, INRA Editions Paris (France), 503-552

Chouinard, P.Y., L.Corneau, D.M. Barbano, L.E. Metzger & D.E. Bauman (1999a). Conjugated linoleic acids alter milk fatty acid composition and inhibit milk fat secretion in dairy cows. Journal of Nutrition, 129, 1579-1584.

Chouinard, P.Y., L. Corneau, A. Sæbø & D.E. Bauman (1999b). Milk yield and composition during abomasal infusion of conjugated linoleic acids in dairy cows. Journal of Dairy Science, 82, 2737-2745

Coppock, C.E., C.H. Noller, S.A. Wolfe, C.J. Callahan & J.S. Baker (1972). Effects of forage-concentrate ratio in complete feeds fed ad libitum on feed intake prepartum and the occurrence of abomasal displacement in dairy cows. Journal of Dairy Science, 55, 783-789.

Davis, C. L. & R.E. Brown (1970). Low-fat milk syndrome. In: A.T. Phillipson (ed.) Physiology of Digestion and Metabolism in the Ruminant. Oriel Press: Newcastle Upon Tyne, UK, 636pp.

DeFronzo, R.A., J.D. Tobin & R. Andres (1979). Glucose clamp technique: a method for quantifying insulin secretion and resistance. American Journal of Physiology, 237, E214-223.

de Deckere, J.M.M. van Amelsvoort, G.P. McNeill & P. Jones (1999). Effects of conjugated linoleic acid (CLA) isomers on lipid levels and peroxisome proliferation in the hamster. British Journal of Nutrition, 82, 309-317.

de Veth, M.J., J.M., Griinari, A.M. Pfeiffer & D.E. Bauman (2004). Effect of CLA on milk fat synthesis in dairy cows: comparison of inhibition by methyl esters and free fatty acids, and relationships among studies. Lipids, 39, 365-372.

de Veth M.J., S.K. Gulati, L.D.Luchini & D.E. Bauman (2005). Comparison of calcium salts and formaldehyde-protected conjugated linoleic acid in inducing milk fat depression. Journal of Dairy Science, 88, 1685-1693.

Doreau, M & Y. Chilliard (1997). Effects of ruminal or postruminal fish oil supplementation on intake and digestion of dairy cows. Reproduction, Nutrition, Development, 37, 113-124.

Drackley, J.K. (1999). Biology of dairy cows during the transition period: The final frontier? Journal of Dairy Science, 82, 2259-2273.

Dunshea, F.R. A.W. Bell & T.E. Trigg (1988). Relations between plasma non-esterified fatty acid metabolism and body tissue mobilization during chronic undernutrition in goats. British Journal of Nutrition, 60, 633-640.

Emery, R.S. (1988). Milk fat depression and the influence of diet on milk composition. Veterinary Clinics of North America: Food Animal Practice, 4, 289-305.

Engvall, A. (1980). Low milk fat syndrome in Swedish dairy cows. Acta Veterinaria Scandinavica, 72, 1-124.

Erdman, R. (1996). Milk fat depression: Some new insights. In: Proceedings of the Tri-State Dairy Nutrition Conference, Fort Wayne, IN, 1-16.

Erdman, R. (1999). Trans fatty acids and fat synthesis in milk. In: Proceedings of the Southwest Nutrition and Management Conference, University of Arizona, Tucson, AZ, 113-125.

Fox, D.G., L.O. Tedeschi, T.P. Tylutki, J.B. Russell, M.E. Van Amburgh, L.E. Chase, A.N. Pell & T.R. Overton (2004). The Cornell net carbohydrate and protein system model for evaluating herd nutrition and nutrient excretion. Animal Feed Science and Technology, 112, 29-78.

Gaynor, P.J., R.A. Erdman, B.B. Teter, J. Sampugna, A.V Capuco, D.R.Waldo & M. Hamosh (1994). Milk fat yield & composition during abomasal infusion of cis or trans octadecenoates in Holstein cows. Journal of Dairy Science, 77, 157-165.

Giesy, J.G., M.A. McGuire, B. Shafii & T.W. Hanson (2002). Effect of dose of calcium salts of conjugated linoleic acid (CLA) on percentage and fatty acid content of milk fat in midlactation Holstein cows. Journal of Dairy Science, 85, 2023-2029.

Godden, S.M., S.C. Stewart, J.F. Fetrow, P. Rapnicki, R. Cady, W. Weiland, H. Spencer & S.W. Eicker (2003). The relationship between herd rbST-supplementation and other factors and risk for removal for cows in Minnesota Holstein dairy herds. In: Proceedings of the Four-State Nutrition Conference. MidWest Plan Service publication MWPS-4SD16. 55–64.

Griinari, J. M. & D.E. Bauman (1999). Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. In: M.P. Yurawecz, M.M. Mossoba, J.K.G. Kramer, M.W. Pariza & G.J. Nelson (eds.) Advances in Conjugated Linoleic Acid Research. AOCS Press, Champaign, IL, 480pp.

Griinari, J.M. & D.E. Bauman (2003). Update on theories of diet-induced milk fat depression & potential applications. In: P.C. Garnsworthy & J. Wiseman (eds.) Recent Advances in Animal Nutrition.. Nottingham, UK, Nottingham University Press, 207pp.

Griinari J.M., P.Y Chouinard & D.E. Bauman (1997). Trans fatty acid hypothesis of milk fat depression revisited. Proceedings of Cornell Nutrition Conference, Cornell University, Ithaca NY, 208-216.

Griinari, J.M., D.A. Dwyer, M.A. McGuire, D.E. Bauman, D.L. Palmquist & K.V.V. Nurmela (1998). Trans-octadecenoic acids and milk fat depression in lactating dairy cows. Journal of Dairy Science, 81, 1251-1261.

Griinari, J.M., K. Nurmela, D.A. Dwyer, D.M. Barbano & D.E. Bauman (1999). Variation of milk fat concentration of conjugated linoleic acid and milk fat percentage is associated with a change in ruminal biohydrogenation. Journal of Animal Science, 77 (Suppl. 1), 117-118 (abstract).

Gröhn, Y.T., S.W. Eicker & J.A. Hertl (1995). The association between previous 305-day milk yield and disease in New York state dairy cows. Journal of Dairy Science, 78, 1693-1702.

Grummer RR. 1991. Effect of feed on the composition of milk fat. Journal of Dairy Science, 74, 3244-3257

Grummer, R.R. (1995). Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. Journal of Animal Science, 73, 2820-2833.

Grummer, R.R. & D.J. Carrol (1991). Effects of dietary fat on metabolic disorders and reproductive performance of dairy cattle. Journal of Animal Science, 69, 3838-3852.

Gulati, S.K., S. McGrath, P.C. Wynn & T.W. Scott (2001). Rumen protected conjugated linoleic acids: effects on milk composition in dairy cows. Proceedings of the Nutrition Society of Australia, 25, S85 (abstract).

Hagemeister, H. (1990). Fetteinsatz in der Milchviehfutterung unter besonderer Berucksichtigung der im Pansen entstehenden trans-Fettsauren. Kieler Milchwirtschaftliche Forschungsberichte, 42, 271-280.

Harfoot, C.G. & G.P. Hazlewood (1988). Lipid metabolism in the rumen. In: P.N. Hobson (ed.) The Rumen Microbial Ecosystem. Elsevier, Amsterdam, pp. 285-322.

Harris, M.A., R.A. Hansen, J.L. Koslo, J.B. Thomas, B.A. Watkins & K.G.D. Allen (2001). Effects of conjugated linoleic acids and docosahexaenoic acid on rat liver and reproductive tissue fatty acids, prostaglandins and matrix metalloproteinase production. Prostaglandins, Leukotrienes & Essential Fatty Acids, 65, 23-29.

Horton, J.D., J.L. Goldstein & M.S. Brown (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. Journal of Clinical Investigation, 109, 1125–1131.

Hove, K. (1978a). Effects of hyperinsulinemia on lactose secretion and glucose uptake by the goat mammary gland. Acta Physiologica Scandinavica, 104, 422-430.

Hove, K. (1978b). Maintenance of lactose secretion during acute insulin deficiency in lactating goats. Acta Physiologica Scandinavica, 103, 173-179.

Hurtaud, C., S. Lemosquet & H. Rulquin (2000). Effect of graded duodenal infusions of glucose on yield and composition of milk from dairy cows. 2. Diets based on grass silage. Journal of Dairy Science, 83, 2952-2962.

Jenny, B.F., C.E. Polan & F.W.Thye (1974). Effects of high grain feeding and stage of lactation on serum insulin, glucose and milk fat percentage in lactating cows. Journal of Nutrition, 104,379-385.

Jump, D.P. (2004) Fatty acid regulation of gene transcription. Critical Reviews in Clinical Laboratory Sciences, 41, 41-78.

Kalscheur, K.F., B.B. Teter, L.S. Piperova & R.A. Erdman (1997). Effect of fat source on duodenal flow of trans-$C_{18.1}$ fatty acids and milk fat production in dairy cows. Journal of Dairy Science, 80, 2115-2126.

Kelley, D.S. & K.L. Erickson (2003). Modulation of body composition and immune cell functions by conjugated linoleic acid in humans and animal models: Benefits vs. risk. Lipids 38, 377–386.

Kramer, J.K.G., N. Sehat, M.E.R. Dugan, M.M. Mossoba, M.P. Yurawecz, J.A.G. Roach, K. Eulitz, J.L. Aalhus, A.L. Schaefer & Y. Ku (1998). Distributions of conjugated linoleic acid (CLA) isomers in tissue lipids of pigs fed a commercial CLA mixture determined by gas chromatography and silver ion-high-performance liquid chromatography. Lipids, 33, 549-558.

Laarveld B, R.K. Chaplin & R.P. Brockman (1985). Effects of insulin on the metabolism of acetate, β-hydroxybutyrate and triglycerides by the bovine mammary gland. Comparative Biochemistry and Physiology , 82B, 265-267.

Loor, J.J., A. Ferlay, M. Doreau & Y. Chillard (2002). Intestinal supply of *trans*-10, *cis*-12 18:2 lowers milk fat output in Holstein cows fed a high- or low-fiber diet with two levels of linseed oil. Journal of Dairy Science 85(Supplement 1), 1189 (abstr.).

Loor, J.J., A. Ferlay, A. Ollier, M. Doreau & Y. Chilliard (2005). Relationship among *trans* and conjugated fatty acids and bovine milk fat yield due to dietary concentrate and linseed oil. Journal of Dairy Science 88, 726-740.

Loor, J.J. & J.H. Herbein (1998). Exogenous conjugated linoleic acid isomers reduce bovine milk fat concentration and yield by inhibiting de novo fatty acids synthesis. Journal of Nutrition, 128, 2411-2419.

Loor, J.J. & J.H. Herbein (2003). Reduced fatty acid synthesis and desaturation due to exogenous *trans*10, *cis*12-CLA in cows fed oleic or linoleic oil. Journal of Dairy Science, 86, 1354-1369.

Lucy, M.C., C.R. Staples, W.W. Thatcher, P.S. Erickson, R.M. Cleale, J.L. Firkins, J.H. Clark, M.R. Murphy & B.O. Brodie (1992). Influence of diet composition, dry matter intake, milk production and energy balance on time of postpartum ovulation and fertility in dairy cows. Animal Production, 54, 323-331.

Mackle, T.R., J.K. Kay, M.J. Auldist, A.K.H. MacGibbon, B.A. Philpot, L.H. Baumgard & D.E. Bauman (2003). Effects of abomasal infusion of conjugated linoleic acid on milk fat concentration & yield from pasture-fed dairy cows. Journal of Dairy Science, 86, 644-652.

Mattos, R., C.R. Staples, A. Arteche, M.C. Wiltbank, F.J. Diaz, T.C. Jenkins & W.W. Thatcher (2004). The effects of feeding fish oil on uterine secretion of PGF_2, milk composition, and metabolic status of periparturient Holstein cows. Journal of Dairy Science, 87, 921-932.

Mattos R., C.R. Staples & W.W. Thatcher (2000). Effects of dietary fatty acids on reproduction in ruminants. Reviews of Reproduction, 5, 38-45.

Mattos, R., C.R. Staples, J. Williams, A. Amorocho & M.A. McGuire (2002). Uterine, ovarian, and production responses of lactating dairy cows to increasing dietary concentrations of menhaden fish meal. Journal of Dairy Science, 85, 755-764.

Mattos, W. & D.L. Palmquist (1977). Biohydrogenation and availability of linoleic acid in dairy cows. Journal of Nutrition, 107, 1755-1761.

McClymont, G.L. & S.Vallance (1962). Depression of blood glycerides and milk-fat synthesis by glucose infusion. Proceecings of Nutritional Society, 21, xli-xlii.

McPherson, R. & A. Gauthier (2004). Molecular regulation of SREBP function: the Insig-SCAP connection and isoform-specific modulation of lipid synthesis. Biochemistry and Cell Biology, 82, 201-211.

Medeiros, S.R., D.E. Oliveira, L.J.M. Aroeira, M.A. McGuire, D.E. Bauman & D.P.D. Lanna (2000). The effect of long term supplementation of conjugated linoleic acid (CLA) to dairy cows grazing tropical pasture. Journal of Dairy Science, 83 (Suppl. 1), 169. (abstract).

Miller, P.S., B.L. Reis, C.C. Calvert, E.J. DePeters & R.L. Baldwin (1991). Patterns of nutrient uptake by the mammary glands of lactating dairy cows. Journal of Dairy Science, 74, 3791-3799.

Molkentin, J. & D. Precht (1995). Optimized analysis of trans-octadecenoic acids in edible fats. Chromatographia, 41, 267-272.

Moore, C.E., H.C. Hafliger III, O.B. Mendivil, S.R. Sanders, D.E. Bauman & L.H. Baumgard (2004). Increasing amounts of conjugated linoleic acid (CLA) progressively reduce milk fat synthesis immediately postpartum. Journal of Dairy Science, 87, 1886-1895.

National Research Council (2001). Nutrient Requirements of Dairy Cattle. National Academy Press, Washington D.C.

Newbold, J.R., K.L. Robertshaw & H.W. Morris (1998). Associations between concentrations of fat and intermediates of ruminal biohydrogenation in milk of dairy cows. Proceedings of British Society of Animal Science, 224 pp (abstract).

Nordlund, K. (1995). Questions and answers regarding rumenocentecis and the diagnosis of herd-based subacute rumen acidosis. In: Proceedings Four State Applied Nutrition and Management Conference, LaCrosse, Wisconsin.

Offer, N.W., M.Marsden, J.Dixon, B.K. Speake & F.F. Thacker (1999). Effect of dietary fat supplements on levels of n-3 polyunsaturated fatty acids, *trans* fatty acids and conjugated linoleic acid in bovine milk. Animal Science, 69, 613-625

Offer, N.W., M. Marsden & R.H. Phipps (2001). Effect of oil supplementation of a diet containing a high concentration of starch on levels of trans fatty acids and conjugated linoleic acids in bovine milk. Animal Science, 73, 533-540.

Opstvedt, J. (1984). Fish fats. In: J. Wiseman (ed.) Fats in Animal Nutrition. Nottingham Easter School in Agricultural Science No. 37, Butterworths, London, 521pp.

Ørskov, E.R., W.P. Flatt, P.W. Moe, A.W. Munson, R.W. Hemken & J.Katz (1969). The influence of ruminal infusion of volatile fatty acids on milk yield, composition and on energy utilization by lactating cows. British Journal of Nutrition, 23, 433-453.

Ostrowska, E., R.F. Cross, M. Muralitharan, D.E. Bauman & F.R. Dunshea (2003). Dietary conjugated linoleic acid differentially alters fatty acid composition and increases conjugated linoleic acid content in porcine adipose tissue. British Journal of Nutrition, 90, 915-928.

Overton, T.R. & M.R. Waldron (2004). Nutritional management of transition dairy cows: strategies to optimize metabolic health. Journal of Dairy Science, 87, E105-119.

Palmquist, D.L., A.D. Beaulieu & D.M. Barbano (1993). Feed and animal factors influencing milk fat composition. Journal of Dairy Science, 76, 1753-1771.

Palmquist, D.L. & W. Mattos (1978). Turnover of lipoproteins and transfer to milk fat of dietary (1-Carbon-14) linoleic acid in lactating cows. Journal of Dairy Science, 61, 561-565.

Palmquist, D.L. & F.L. Schanbacher (1991). Dietary fat composition influences fatty acid composition of milk fat globule membrane in lactating cows. Lipids, 26, 718-722.

Park, Y., K.J. Albright, J.M.Storkson, W. Liu, M.E. Cook & M.W. Pariza (1999). Changes in body composition in mice during feeding and withdrawal of conjugated linoleic acid. Lipids, 34, 243-248.

Pegorier, J.P., C. May & J. Girard (2004). Control of gene expression by fatty acids. Journal of Nutrition,134,2444S-24449S.

Pennington, J.A. & C.L. Davis (1975). Effects of intra-ruminal and intra-abomasal additions of cod-liver oil on milk fat production in the cow. Journal of Dairy Science, 58**,** 49-55.

Perfield II, J.W., G. Bernal-Santos, T.R. Overton & D.E. Bauman (2002). Effects of dietary supplementation of rumen-protected conjugated linoleic acid in dairy cows during established lactation. Journal of Dairy Science, 85, 2609-2617.

Perfield II, J.W., P. Delmonte, A.L. Lock, M.P. Yurawecz & D.E. Bauman (2004b). *Trans* -10, *trans* -12 conjugated linoleic acid (CLA) reduces the Δ^9-desaturase index without affecting milk fat yield in lactating cows. Journal of Dairy Science, 87 (Supplement 1), 297. (abstract).

Perfield II, J.W., A.L. Lock, A.M. Pfeiffer & D.E. Bauman (2004a). Effects of amide-protected and lipid-encapsulated conjugated linoleic acid (CLA) supplements on milk fat synthesis. Journal of Dairy Science, 87, 3010-3016.

Perfield II, J.W., A. Saebo & D.E. Bauman (2004c). Use of conjugated linoleic acid (CLA) enrichments to examine the effects of *trans*-8, *cis*-10 CLA, and *cis*-11, *trans*-13 CLA on milk fat synthesis. Journal of Dairy Science, 87, 1196-1202.

Peterson, D.G., L.H. Baumgard & D.E. Bauman (2002). Short Communication: Milk fat response to low doses of *trans*-10, *cis*-12 conjugated linoleic acid (CLA). Journal of Dairy Science, 85, 1764-1766.

Peterson, D.G., E.A. Matitashvili & D.E. Bauman (2003). Diet-induced milk fat depression in dairy cows results in increased *trans*-10, *cis*-12 CLA in milk fat and coordinate suppression of mRNA abundance for mammary enzymes involved in milk fat synthesis. Journal of Nutrition, 133, 3098-3102.

Peterson, D.G., E.A. Matitashvili & D.E. Bauman (2004). The inhibitory effect of *trans*-10, *cis*-12 CLA on lipid synthesis in bovine mammary epithelial cells involves reduced proteolytic activation of the transcription factor SREBP-1. Journal of Nutrition, 134, 2523-2527.

Piepenbrink, M.S., A.L. Marr, M.R. Waldron, W.R. Butler, T.R. Overton, M.Vázquez-Añón & M.D. Holt (2004). Feeding 2-hydroxy-4-(methylthio)-butanoic acid to periparturient dairy cows improves milk production but not hepatic metabolism. Journal of Dairy Science, 87, 1071-1084.

Piperova, L.S., J.Sampugna, B.B. Teter, K.F. Kalscheur, M.P. Yurawecz, Y. Ku, K.M. Morehouse & R.A. Erdman (2002). Duodenal and milk trans octadecenoic acid and conjugated linoleic acid (CLA) isomers indicate that postabsorptive synthesis is the predominant source of cis-9-containing CLA in lactating dairy cows. Journal of Nutrition, 132, 1235-1241.

Piperova, L.S., B.B. Teter, I. Bruckental, J. Sampugna, S.E. Mills, M.P. Yurawecz, J. Fritsche, K. Ku & R.A. Erdman (2000). Mammary lipogenic enzyme activity, trans fatty acids and conjugated linoleic acids are altered in lactating dairy cows fed a milk fat-depressing diet. Journal of Nutrition, 130, 2658-2574.

Pullen, D.L., D.L. Palmquist & R.S. Emery (1989). Effect on days of lactation and methionine hydroxy analog on incorporation of plasma fatty acids into plasma triglycerides. Journal of Dairy Science, 72, 49-58.

Reynolds, C.K., P.C. Aikman, B. Lupoli, D.J. Humphries & D.E. Beever (2003). Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. Journal of Dairy Science, 86, 1201-1217.

Rigout**,** S., C. Hurtaud, S. Lemosquet, A. Bach, and H. Rulquin (2003). Lactational effect of propionic acid and duodenal glucose in cows. Journal of Dairy Science, 86, 243-253.

Rigout, S., S. Lemosquet, A. Bach, J.W. Blum & H. Rulquin (2002). Duodenal infusion of glucose decreases milk fat production in grass silage-fed dairy cows. Journal of Dairy Science, 85, 2541-2550.

Schäfer, K., K. Männer, A Sagredos, K. Eder & O. Simon (2001). Incorporation of dietary linoleic and conjugated linoleic acids and related effects of laying hens. Lipids, 36, 1217-1222.

Sébédio, J.-L., E. Angioni, J.M. Chardigny, S. Gregoire, P. Juaneda & O. Berdeaux (2001). The effect of conjugated linoleic acid isomers on fatty acid profiles of liver and adipose tissue and their conversion to isomers of 16:2 and 18:3 conjugated fatty acids in rats. Lipids, 36, 575-582.

Sébédio, J.L., J.M. Chardigny & O. Berdeaux (2003). Metabolism of conjugated linoleic acids. In: J.L. Sébédio, W.W. Christie & R.O. Adlof (eds.) Advances in Conjugated Linoleic Acid Research, Vol. 2. AOCS Press, Champaign, IL, 337pp.

Sébédio, J.-L., P. Juaneda, G. Dobson, I. Ramilison, J.C. Martin, J.M. Chardigny & W.W. Christie (1997). Metabolites of conjugated linoleic acid (CLA) in the rat. Biochimica et Biophysica Acta, 1345, 5-10.

Selner, D.R. & L.H. Schultz (1980). Effects of feeding oleic acid or hydrogenated vegetable oils to lactating cows. Journal of Dairy Science, 63, 1235-1241.

Shingfield K.J., D.E. Beever, C.K. Reynolds, S.K. Gulati, D.J. Humphries, B.Lupoli, G. Hervás & J.M. Griinari (2004). Effect of rumen protected conjugated linoleic acid on energy metabolism of dairy cows during early to mid-lactation. Journal of Dairy Science 87 (suppl.1), 307 (abstract).

Staples, C.R., J.M. Burke & W.W. Thatcher (1998). Influence of supplemental fats on reproductive tissues and performance of lactating cows. Journal of Dairy Science, 81, 856-871.

Strang, B.D., S.J. Bertics, R.R. Grummer & L.E. Armentano (1998). Effect of long-chain fatty acids on triglyceride accumulation, gluconeogenesis and ureagenesis in bovine hepatocytes. Journal of Dairy Science, 81, 728–739.

Sutton, J.D. (1985). Digestion and absorption of energy substrates in the lactating cow. Journal of Dairy Science, 68, 3376-3393.

Sutton, J. D., I. C. Hart, S. Morant, V. Schuller & A. D. Simmonds (1988). Feeding frequency for lactating cows: diurnal patterns of hormones and metabolites in peripheral blood in relation to milk-fat concentration. British Journal of Nutrition, 60, 265-274.

Terpstra, A.H.M., A.C. Beynen, H. Everts, S. Kocsis, M.B. Katan & P.L. Zock (2002). The decrease in body fat in mice fed conjugated linoleic acid is due to increases in energy expenditure and energy loss in the excreta. Journal of Nutrition, 132, 940-945.

Van Soest, P.J. (1963). Ruminant fat metabolism with particular reference to factors affecting low milk fat and feed efficiency. A review. Journal of Dairy Science, 46, 204-216.

Van Soest, P.J. (1994). Nutritional Ecology of the Ruminant. Cornell University Press, Ithaca, NY.

Vernon, R.G. & S. Sasaki (1991). Control of responsiveness of tissues to hormones. In: T. Tsuda,Y. Sasaki & R. Kawashima (eds.) Physiological Aspects of Digestion and Metabolism in Ruminants. Academic Press, San Diego, CA, 779pp.

Virtanen, A.I. (1966). Milk production of cows on protein-free feed. Science, 53, 1603-1614.

West, D.B., J.P Delany., P.M. Camet, F. Blohm, A.A. Truett & J. Scimeca (1998). Effects of conjugated linoleic acid on body fat and energy metabolism in the mouse. American Journal of Physiology, 44, R667-R672.

Wolf, E., G.J. Arnold, S. Bauersachs, H.M. Beier, H. Blum, R. Einspanier, T. Frohlich, A. Herrler, S. Hiendleder, S. Kiolle, K. Prelle, H.-D. Reichenbach, M. Stojkovic, H. Wenigerkind & F. Sinowatz (2003). Embryo-maternal communication in bovine – strategies for deciphering a complex cross-talk. Reproduction of Domestic Animals, 38, 276-289.

Wonsil, B.J., J.H. Herbein & B.A. Watkins (1994). Dietary and ruminally derived trans-18:1 fatty acids alter bovine milk lipids. Journal of Nutrition, 124, 556-565.

Zhao F-Q, W.M. Moseley, H.A. Tucker & J.J.Kennelly (1996). Regulation of glucose transporter gene expression in mammary muscle, and fat of lactating cows by administration of bovine growth hormone and bovine growth hormone-releasing factor. Journal of Animal Science, 74, 183-89.

Part VI: Nutrition and immunology

Endocrine effects on immune function: defining opportunities based on knowledge from growing calf and periparturient animal models

T. Elsasser[1], K.L. Ingvartsen[2], S. Kahl[1], and A.V. Capuco[1]
[1]USDA-Agricultural Research Service, Beltsville, Maryland 20705, USA
[2]Danish Institute of Agricultural Science, Dept. of Animal Health and Welfare Research Centre Foulum , Tjele, Denmark

Abstract

Birth and parturition, lactation, weaning, castration, and seasonal diet change constitute defined, and, for the most part, naturally occurring periods in animals' lives where a needed priority for a specific biological function temporarily challenges the stability of other physiological processes. A tremendous quantity of data has been amassed in attempts to categorize nutritional, hormonal, and immunological perturbations associated with these stresses. However, clear cause and effect relationships remain elusive and thus biochemically-defined, pathway-based decision structures towards preventive intervention lack in favor of the more traditional treatment of symptoms. Many of the physiological conflicts of this sort can be appreciated in the delicate, but critically balanced state of the periparturient dairy cow and the need to recognize the importance of individual animal variability in the management scheme. We present here some newer findings and implications regarding endocrine-immune interactions and effects suggesting targets towards which intervention strategies could be developed for physiological critical periods. As examples of principle, the ability for growth hormone to critically regulate at multiple points the activity of nitric oxide synthases in the immune response to inflammation and the metabolic fate of nitric oxide are discussed. Similarly, the differential regulation of multifunctional peptides like adrenomedullin is presented.

Keywords: hormone, cytokine, stress, homeostasis, intervention

Introduction

It is more accurate to address the topic of endocrine system regulation of immune function as an interaction of systems rather than a simple regulation because of the complex feedback and interplay that exists at the cellular level where immune, endocrine and nutritional signals converge to direct cell function. In discussing the role of endocrine system interactions with the immune system in specific regard to the periparturient dairy cow, many cause-and-effect relationships that ordinarily flow somewhat logically, as an effect of an endocrine hormone on some immunological property and response, are clouded by the consequences of the conflicts in biological priority that surround the periparturient time in the cow. Trying to summarize the many places that each axis of the endocrine system (reproductive, thyroid, pancreatic, hypothalamic-pituitary, gut, or adipose) could impact immune function in the periparturient cow might serve to confuse rather than enlighten the reader because at this time what we can document regarding these

interactions are more reflective of compensatory metabolic responses to management factors that we impose on the cow at this time, than true cause and effect relationships. The purpose of this review therefore is to highlight certain specific endocrine factors that explicitly underlie the immune status of the cow at this time and furthermore suggest critical control points at which we can direct intervention strategies to make it easier for the cow to balance the biological priorities and tasks asked of her. Therefore, following a brief review of some management factors that impact the metabolic status of the cow during the periparturient time, focus will be on (a) the somatotropic axis [growth hormone (GH) and insulin-like growth factor-1 (IGF-1)] effects that impact on aspects of immune function, (b) endocrine input to immunochemical pathway-based critical control points in tissues where an imbalance in the intracellular generation of reactive oxygen and nitrogen compounds can establish a harmful condition, and (c) features of a newly discovered peptide hormone, adrenomedullin, that, because of its functions as a regulator of metabolism, apoptosis, vascular tone, modulation of immune system-directed nitric oxide production and complement cascade and, finally, novel character as an antimicrobial peptide, place it in a pivotal role as a directly linking the endocrine and immune systems both in states of health and disease.

Brief overview of the periparturient phenomenon

Dairy cows are reared for the explicit purpose of producing calves and milk and to accomplish this we manage the reproductive life of the animal. In a discussion of interactions between the endocrine and immune systems in the periparturient cow the first task at hand is to define the nature of the animal we are really considering. Dairy cows are specifically managed during the time period surrounding parturition to obtain a mammary tissue matrix in the subsequent lactation capable of redelivering a large milk output. To accomplish this dairy cows are dried off prior to calving and this process, as it is presently used, involves a timed reduction in feed, cessation in milking and, in some contries, the administration of antibiotics to offset immunological difficulties in maintaining defensive barriers to infection, particularly mammary infection, as well as many well-characterized metabolic disturbances also commonly associated with increased propensity for the cow to fail to adequately fight off infection (for reviews of the nutrient management of the periparturient- transition dairy cow please see: Sorensen and Enevoldsen, 1991; Enevoldsen and Sorensen, 1992; Oetzel, 2000; Dingwell *et al*., 2003). It is intuitive that some degree of homeostatic challenge is imparted to the dairy cow in these management practices. Based on observations in beef cows where the overall level of immunoincompetance is relatively mild compared to that of the dairy cow where beef cows are not managed in the same format for drying off (Wren, 2003). There are definite differences in the susceptibility to infection, albeit there are factors such as efficiency of disease detection, level of milk production, genetics, and environment that influence this observation.

Physiologists have long sought to compartmentalize individual systems largely to bring forth some degree of clarity, focus, and purpose to the tremendously complex cellular functions and interactions. With reference to our purpose here, the early categorizations of an "endocrine system" and an "immune system" forced a largely artificial separation of function that we now realize are so intricately intertwined that the terms "peptide hormone" and "cytokine" are largely synonymous and interchangeable. Hormones and cytokines exist in a biochemical world for the

sole purpose of regulating the body's internal processes to satisfy the need for a species to survive and propagate. In accomplishing these tasks, priorities for biological processes exist in the body and the relationships between the priorities change throughout life as the need to grow to sexual maturity is supplanted by the need to actively reproduce. The single largest challenge to the stability and progression through these metabolic priorities lies in the changing, and sometimes fragile, capacity to compensate for and counteract distractions, ie., stresses, that open the door wider for opportunistic disease vectors to divert tissues from their physiological mission. Thus a main difficulty in defining the impact of the endocrine system on immune function is the fact that the rules governing the relationships change between states of relative health and stress. At present, the guiding principle of endocrine regulation of immune function can be summarized as a "temporally defined modulation of orchestrated immune system shifts" rather that what has been historically patterned as either enhancement or attenuation of a response.

The periparturient dairy cow's physiology has become the center of focus in regard to how fragile the balance is between biological processes (Goff and Horst, 1997; Ingvartsen and Andersen, 2000; Burton *et al*., 2001). Fetal developmental needs, lactation initiation, and the immuno-surveillance to maintain cow/calf health status are now finely balanced against the demands imposed on these systems to facilitate increased milk production as domestication of these animals has progressed to the point where they are truly "metabolic athletes". The high level of production characteristic of modern dairy cattle has been questioned in terms of how significant the impact is on animal health, given the extent of disease incidence recorded for cattle during the periparturient period. A critical review of international data compiled from epidemiological and genetic studies indicated that the only strong correlation between milk yield and disease incidence was that associated with mastitis (Ingvartsen *et al*., 2003). In regards to other metabolic diseases associated with this time period, these investigators concluded that while tendencies could be interpreted to suggest a higher incidence of dystocia, retained placenta, metritis, displaced abomasum or ketosis, lameness and periparturient paresis in higher yielding animals, there is no objective documentation to assess this at this time. They proposed the metabolic rubric outlined in Figure 1 to structure potential avenues of interaction between metabolic state, genetics and the environment that could culminate in a higher incidence of disease in these animals. Significant to the interactions is the fact that compartmentalized metabolic priorities change during this period due to the need to "finish" fetal development, initiate lactation and channel nutrients in limited supply to tissues of high metabolic priority. If one utilizes the readily accessible availability of biomarker sampling from the circulatory compartment we can easily define many of the metabolic perturbations that exist at this time in terms of altered nutrient, metabolite and hormone concentrations that mirror the state created where tissue reserves are mobilized to compensate for the nutrient and caloric deficits accompanying inadequate voluntary intake.

Hormonal maintenance of pregnancy and the impact of fetal-maternal tolerance on natural immunosuppression

Some degree of distinction needs to be mentioned regarding elements of immunosuppression surrounding the periparturient period in the dairy cow. Literature as well as veterinary clinical accounts clearly indicates that the dairy cow exists around the time of parturition in what develops as an immunologically compromised state where opportunities exist for infection to gain an

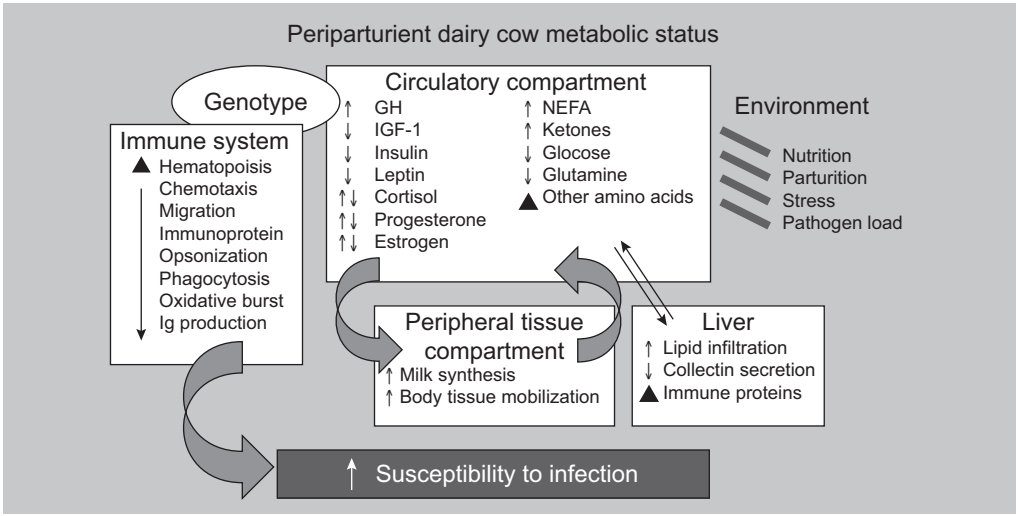

Figure 1. Periparturient changes in hormones, metabolites, immune factors and health status reflect the integration of signals needed to redirect metabolism from a pregnancy state to a lactation state where the gene -by-environment interactions, including intended management decisions, play a significant role in shaping the intensity of pressures that might lead to stress-impaired homeostatic balance (Source: Ingvartsen et al.*, 2003).*

upper hand on the body and fulminate into active disease. Immunologists have documented several aspects of immune function that participate in this characterization. Notable are such factors as (a) leukocyte gene repression (Madsen *et al*., 2002), altered phagocytic and oxidative burst capacity and numbers of neutrophils and macrophages (Burvenich *et al*., 2004; Meglia *et al*., 2001), altered neutrophile:lymphocyte ratios (Jacob *et al*., 2001) and altered efficiency of antibody response (Kehrli *et al*., 1999; Mallard *et al*., 1997; Mallard *et al*., 1998). We can characterize this immunosuppression in terms of (a) generalized systemic and (b) organ-centric with certain degrees of overlapping interactions that influence the function of the alternate compartment. In particular, much of the immunosuppression might be accounted for in underachieving a switch from TH-1 proinflammatory to TH-2-type immune responses (Kherli *et al*., 1999; Pfaffl *et al*., 2003). For example, estrogen and progesterone status lie at the heart of the immunomodulatory state locally (organ-centric) directed within the fetal-placental unit as dictated by their needed actions in the physiological process that command the highest priority at this time, the generation and development of the fetal calf lasting to term. Estrogen(s) appear to have suppressing activity in TH1-type proinflammatory and an enhancing impact on cell mediated TH2-type immune responses (Salem, 2004). Similarly, progesterone has been characterized as immunosuppressive (Koch and Platt, 2003; Stites and Siiteri, 1983), necessary for evading fetal rejection, and a participating trigger (falling levels of progesterone) along with fetal-directed prostaglandin release and altered placentome steroid receptor functionality (Boos, *et al*., 2000) in the initiation of parturition in the cow.

Conception and pregnancy are essentially times where the dam reexamines her immunological surveillance priorities to permit the establishment of a "foreign" allograft internally, so to speak, the ultimate immunocompromized state (Gluckman *et al.*, 1999; Gorczynski *et al.*, 2002). Initiated at conception, fetal – maternal tolerance (Rogers *et al.*, 1998) constitutes a progesterone-driven series of events, mainly localized to the uterine-placental structure, to suppress what would amount to allograft rejection to maintain the growth of nonautologous tissue (Chaouat, 1987), what Bazer and First (1983) termed a "privileged site". Active in this process, and very indicative of the extent of endocrine and immune system interactions, is the hormonal control of decidual cell and trophoblast activity that elicits a milieu of cytokine (interleukin-1, -2)-driven suppressor molecules necessary to thwart fetus rejection and abortion. Participating components of the process include modulation of reactivity towards major histocompatability complex (MHC-) alloantigens, changes in the production of and sensitivity to alpha-fetoprotein and alpha-2-macroglobulin, increased localization of immunosuppressor cells in the decidua, cytokine-directed tissue remodeling, and blocking of the complement-mediated cytolytic cascade. The capacity for the fetus to communicate to the maternal tissues via its own hypothalamic-pituitary-adrenal components further modulates maternal immune responses and the fetal release of glucocorticoids constitutes a major signal for prostaglandin-facilitated initiation of labor (Challis *et al.*, 2001; Schwartz and McMillen, 2001).

Somatotropic axis modulation of immune function

Certainly within the construct of endocrine influences on immune function, the hypothalamic-pituitary unit holds a prominent position in its ability to integrate peripheral as well as central signals regarding the stability of the internal milieu and governs the delivery of most classical endocrine hormones to sites of action. Probably the most studied axis in this regulatory scheme is the hypothalamic-pituitary-adrenal component historically referred to as "immunosuppressive' in its actions where increased levels of glucocorticoids were thought to play a major role in downregulating immune function, proinflammatory function in particular. Particularly recent data summarized by Elenkov (2004) suggest that the glucocorticoid release associated in particular with the onset of the acute phase response modulates rather than decreases immunological responsiveness in regard to the balance between TH1 and TH2 responses (Sternberg, 2001), limiting the potential for the immune system to "overshoot" in the TH-1-proinflammatory response period resulting in added tissue stress and disease. Because of the extensive nature of the research dealing with this particular aspect of immune regulation, this review will defer to other summarizations of these effects (Burvenich *et al.*, 2004; Diez-Fraile *et al.*, 2003) and yield to opportunities to discuss newly appreciated interactions through which the endocrine influences modulate immune response.

From a systemic perspective, many of the effects of endocrine function that impact immune function during the periparturient period may be described in part through how the somatotropic axis responds to the demands of the different levels of metabolic stress that develop at this time. The reason that this is of interest in this periparturient model largely stems from the fact that the somatotropic axis plays such an important role in the needed coordination of nutrient trafficking as dynamic changes in tissue priorities evolve with the transition from a pregnant to a lactating dam. Combine this with the fact that we further manage the cow in a dry state (Dingwell *et al.*,

2003, Goff and Horst, 1997), and suddenly we are faced with a multiplicity of biological needs all needing a nutrient supply that is, at best, also compromised in terms of energy and protein The major elements of the somatotropic axis, GH and IGF-1, serve as a pivotal integrating resource in terms of homeostatic and homeorhetic control by their response to nutrient availability, their direct impact on immune processes, and in large part, their impact on the direct local tissue effectors like nitric oxide and superoxide anion that further regulate cell function.

The regulatory interactions between GH and IGF-1 were easy to comprehend during earlier time periods when the prevailing concept was that GH regulated IGF-1 production by tissues, the liver in particular, and the ability to do so was more or less a permissive effect dictated by the plane of nutrition. In the classical view of IGF-1 regulation, inadequate levels of dietary protein and energy were associated with what was termed the "uncoupling" of the somatotropic axis where it could be demonstrated that GH failed to stimulate production of IGF-1, IGF-1 plasma levels declined, and basal GH concentrations increased (Elsasser *et al.*, 1989; McGuire *et al.*, 1998). In recent years, it has become more apparent that the regulation of IGF-1 in various tissues has additional elements of paracrine and autocrine control, with many hormone-directed actions largely not reflected in systemic changes in plasma concentrations of GH or IGF-1 (Pfaffl *et al.*, 1998; Elsasser, 2003). Similarly, whereas liver content of mRNA for IGF-1 was affected by nutritional status, gut, muscle and skin levels of IGF-1 mRNA were unaffected by plane of nutrition, with the speculation that a more sophisticated role for IGF1 in these tissues was present, perhaps a role more aligned with localized tissue health and homeostasis (Adams *et al.*, 2000). However, because a given level of hormone in plasma is only as good as the quality of the signal transduction processes that allow cells to respond to the message, the modulation of receptor function, accessibility of hormones to tissues via transport binding proteins, hormone flux and regional blood flow, and intracellular signal transduction processes, stand to significantly shape the nature of the immune response to a so-called endocrine process (Elsasser and Kahl, 2002).

Presently, the relationship between GH and IGF-1 and nutrition can only be described in context to a particular tissue of interest at a given time. Localized paracrine production of these hormones, in particular by immune system elements and tissues responding locally to immunological processes, can dramatically shape the metabolic and immune character of a very discrete tissue area, in contrast to so many of the generalizations we have documented with systemic measurement of hormone and cytokine changes. For example, dietary protein or energy content and intake have been regarded as factors contributing to the regulation of IGF-1 production in cattle (Elsasser *et al.*, 2000; Bauman 2000), certainly as it has been defined with measurements of plasma IGF-1. With the development of technologies permitting the site-specific inhibition or overproduction of IGF-1 (ie., IGF-1 knockout and liposome-mediated gene transfer, Dasu *et al.*, 2003), we now realize the greater dynamic relationship that exists in the regulation of and interactions between immune system components and IGF-1, in particular as regards not only the immediate concentrations of hormone in the region but also in terms of the propensity for tissues to reestablish their sensitivity to a hormones. Recently, a research team using liver-specific IGF-1 knockout mice reported a significant increase in lymphoid (B-lymphocytes) receptor presence for GH and IGF-1 during protein-calorie malnutrition suggesting a modulation in the tissue sensitivity and responsiveness to GH and IGF-1 to maintain the tissue homeostasis and function at the local level more or less independent of the prevailing plasma hormone level (Naranjo *et*

al., 2002). Similarly Dasu *et al*. (2003) successfully demonstrated the propensity for liposome-mediated gene transfer to deliver increased IGF-1 locally to complement the immunological response needed in the restoration of wound healing.

Pioneering work on the effects of GH and IGF-1 on neutrophil and monocyte macrophage functions including phagocytosis and oxidative burst activity, as well as antibody production was made possible with the generation of recombinant peptide material. This work clearly demonstrated that several effects of GH and IGF-1 were associated with overall improved immune function and disease resistance (Kelley, 1990; Johnson *et al*., 1997; van Buul-Offers and Kooijman, 1998; Heemskerk *et al*., 1999) and regulation of innate immunity via GH effects on mannan binding lectin (Hansen, 2003). With new information regarding the infiltration and sequestering of mobile immune cells such as the neutrophils and monocyte/macrophages, we now need to account for location-specific activity changes in these immune cells as directed by the local production of IGF-1 by the tissues resident in the affected area. Even the metabolic effects of GH and IGF-1 to modulate adipose tissue function can be considered as an immunoregulatory role in that newer findings clearly demonstrate that paracrine effects of adipocytes on juxtaposed lymphoid cells are effected by the fat cell release of cytokines (Coppack, 2001) and a direct effect of fat cell-derived leptin on circulating immune cells (Ingvartsen and Boisclair, 2001). In this regard, the relative priority for resource (nutrient) partitioning to fortify the increased metabolic needs of the immune cells can also be directed and accounted for. Interestingly, this capability further highlights the nature of the bidirectional communication between immune system and endocrine effectors wherein the capability for lymphocytes to secrete GH may participate in the localized production of tissue-derived IGF-1 and therefore the capability for the activities of the immune cells to be further regulated via IGF-1. The generalized effects of GH and IGF-1 on immune function are summarized in Table 1.

Temporal, state-dependant, and inter-animal variability factors in the endocrine control of immune function

Still, other distinctions need to be made to accurately address the implications of endocrine influences on immune function. Again, defaulting back to the influences of GH or the estrogen-progesterone status of an animal on immune function, we can observe that sometimes the immune system needs to be activated, or activated in a particular manner, in order for either a significant impact of a hormone to be observed. Under healthy basal conditions we might be limited by the sensitivity of the analytical method to detect meaningful changes in an immune parameter. Estrogen-progesterone treatment of steers resulted in a significant increase in spontaneous and specific mitogen (ovalbumin)-directed blastogenesis and the CD4/CD8 cell ratio, an observation lost in the background of generalized blastogenic response to generalized mitogens like PHA (Burton *et al*., 1993). More recent data in this area suggest that the observed effects may be mediated through a steroid-driven modulation of nitric oxide production by nitric oxide synthase and thromboxane-B_2 (Sartin *et al*., 2003).

Historically, a role for GH as an immune modulator extended from the observations that treatment of experimental animals with GH resulted in increased thymus weight and augmented thymic function (Kelley, 1990). Since these early observations, the impact of GH and IGF-1 on immune

Table 1. Effects of GH, prolactin, and IGF-1 on general immune system functions (Source: Information summarized from: Elsasser et al.*, 1994; vanBuul-Offers and Kooijman, 1998; Burgess* et al.*, 1999; Heemskerk* et al.*, 1999; Dorschkind and Horseman, 2000; Dorschkind and Horseman, 2001; Dasu* et al.*, 2003).*

GH and IGF-1:	
	Generalized role in immune system – protective/homeostatic stability
	Anabolic to immune system cells/ stress modulators in collaboration with adrenal response axis
	IGF-1 and prolactin antiapoptotic in immune cells – caspase3, BAX- anti-inflammatory NF-kappa B and activation protein (AP)-1 transcription factors
	Prolactin counter-regulatory to adverse lymphoid apoptotic actions of high glucocorticoid states
GH deficiencies:	
	Reduced thymulin production and release
	Thymic atrophy
	Delayed antibody synthesis
	Delayed tissue graft rejection
	Reduction in T and B cell numbers and functionality
	Reduced activity of natural killer (NK) cells
GH and IGF-1 supplementation:	
	Increased thymic activity
	Regeneration of age-related thymic atrophy
	Increased thymulin production
GH effects on lymphoid cells:	
	Modulated activity and metabolism of lymphocytes, neutrophils, and monocyte-macrophages via GH and IGF-1 receptors
	Paracrine relationships vis immune cell-derived GH and IGF-1
	Increased NK cell activity, IL-2 production, T-cell proliferation
GH/IGF-1 effects on phagocytic cells:	
	Prime for increased superoxide anion burst
	Restoration of respiratory burst in states of GH deficiency
	Modulatory impact on proinflamatory cytokine release
GH interactions with non-lymphoid tissue response to proinflammatory cytokines:	
	GH modulates TNF-α receptor density in hepatic membranes
	GH downregulated arginase / increased nitric oxide substrate sparing
	Increased xanthine oxidase activity/ increased superoxide anion generation
	Modulatory effects on CAT-2 amino acid transporter systems/ arginine flux
	Increased nitric oxide release to proinflammatory challenge via AKT/protein
	Kinase B phosphorylation activation of endothelial nitric oxide synthase

function has become more clearly appreciated. However, as technology and concepts evolved, further research into direct and indirect effects of GH and IGF-1 on immune system function has yielded several important findings. In general terms, the greatest impact of somatotropic axis hormones on immune function may reside in aspects of metabolic and proliferative control

GH and IGF-1 impart to immune cells. Even in terms of the cellular readjustments needed to initiate and maintain pregnancy, both locally produced as well as peripherally-delivered GH and IGF-1 coordinate several placental activities associated with angiogenesis, hormone sensitivity, and immune cell function (Evain-Brion and Malassine, 2003).

With regard to some impact of GH on immune function, we reported first in cattle that the administration of GH to steers has an apparent beneficial effect on the severity of host response to an endotoxin challenge (an immune system challenge model highly repeatable in its effectiveness to rapidly shift from a basal state to a highly reactive proinflammatory state, Elsasser *et al*., 1994). The beneficial effect was mainly brought about through a significant effect of GH to blunt the release of the proinflammatory cytokine tumor necrosis factor-α, cortisol and thromboxane B_2, while decreasing the hepatic tissue content of TNF-α receptors. In a more recent subsequent study, we did not observe the previous finding, but rather saw no effect of the GH treatment on plasma TNF-α, though several inflammatory parameters downstream from the TNF-α response were augmented (Elsasser *et al*., 2004). The discrepancy between these two studies lay in the experimental procedure wherein for the first experiment, GH was administered for only 3 to 5 days prior to endotoxin challenge whereas in the second study, GH was given for 10 to 12 days (Figure 2). However, where actions of GH within the TNF-α response could be demonstrated on endotoxin–challenged cattle, little or no effect of GH was evident on basal, pre-challenge plasma concentrations of TNF-α, though significant influence on basal TNF levels were apparent with a change in feeding level or implantation with the estrogen progesterone growth modulator Synovex-S (Elsasser *et al*., 1997). Similarly in lactating dairy cattle, Capuco demonstrated that

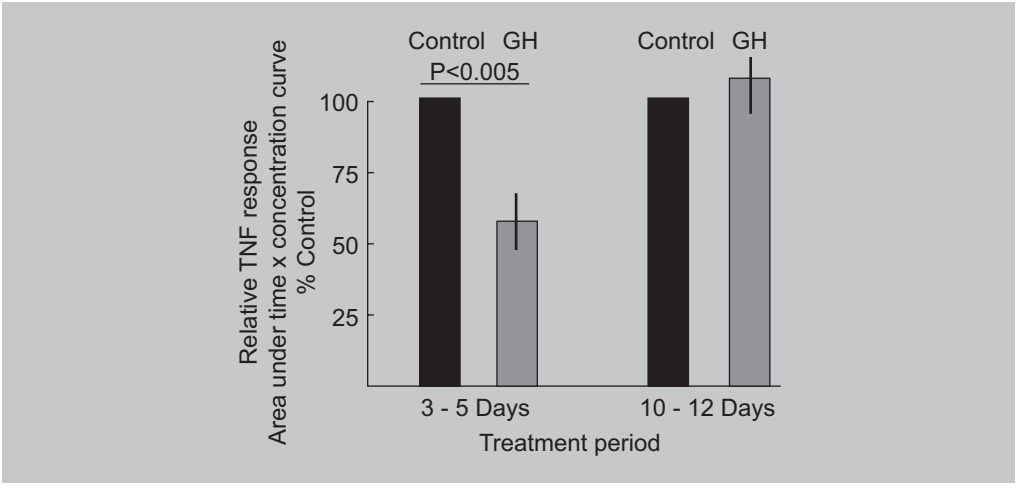

Figure 2. Effects of GH treatment duration on the capacity to effect a down regulation of proinflammatory cytokine release in vivo. Recombinant bovine GH was administered at 0.1 mg/kg, subcutaneously to heifers or steers (average body weight=316 kg) for 3-5 or 10-12 days. The loss of effect after 10 days appears to represent an accommodation of the system wherein the secretory response was no longer impacted by the increased GH status.

a 6 day regimen of GH had no measurable effect on basal TNF levels in plasma (Capuco *et al*., 2001).

Collectively, the data suggest that some of the effects of GH on cell response to inflammatory challenge may manifest themselves differently over a given treatment time frame. Further substantiating this concept of temporally divergent response to GH treatment, when the arginine metabolizing enzyme arginase was measured in liver extracts from all of these animals in the two studies, it was apparent that GH decreased arginase activity in the longer treated cattle while not affecting arginase in the short-term treated cattle. Arginine is critical to immune tissue response because it is also the substrate for the nitric oxide synthase enzymes that generate nitric oxide.

With regard to the periparturient animal, the development of the immunocompromised state and its relationship to endocrine-somatotropic axis status may not be totally straight forward in that there appears to be significant variability between animals as they present themselves as compromised as well as the degree to which different parts of the immune system are affected. How the endocrine system may play a role in the source of variability is being studies by several laboratories and data suggest significant positive correlations between enhanced somatotropic axis function and improved immune responsiveness where defined in terms of efficiency of antibody production (Hernandez *et al*., 2003; Mallard *et al*., 1997; Mallard *et al.,* 1998). Where periparturient dairy cows could be stratified according to intensity of antibody response, high responding animals were less likely to develop disease in contrast to animals that showed poor antibody response. Work continues along these lines as newer technologies are developed and validated in terms of cDNA microarrays to specifically integrate endocrine-immune responses.

A final comment should be made here regarding GH impacts on immune function and the greater role of the endocrine system in modulating immune function in general. Because of the sensitive nature of the metabolic hormone cascade to restructure metabolic priorities, many of the effects of the endocrine system on immune function should be interpreted with regard to the prevailing metabolic status of the experimental animal. Not only plane of available nutrition but also the nutrition further associated with gut uptake as well as necessary energy expenditures should be taken into consideration. For example, many experimental paradigms utilize the maintenance fed animal. If, as determined by Rumsey and Hammond (1990), maintenance energy of implanted animals increases, as reflected in lower rates of tissue deposition in the maintenance fed animals than in unimplanted animals, is the endocrine response "perturbed"? Similarly, in cattle where the capacity to adjust for the augmented energy expenditure associated with increasing surface area ectoparasite infestation with *Psoroptes* sp. (Cole and Guillot, 1987) could be compensated for with increased voluntary intake. But when intake can no longer provide the needed nutrients, does the endocrine balance change to reflect reprioritized physiological needs? In experiments aimed at assessing the impact of intake on the character of the proinflammatory response to endotoxin challenge, we observed that at approximately the same level of intake that would trigger the "uncoupling" of the somatotropic axis where GH would no longer stimulate increases in plasma IGF-1 levels (Elsasser *et al*., 1989), cattle failed to develop the natural tolerance response to repeated endotoxin challenge (Elsasser *et al*., 1997).

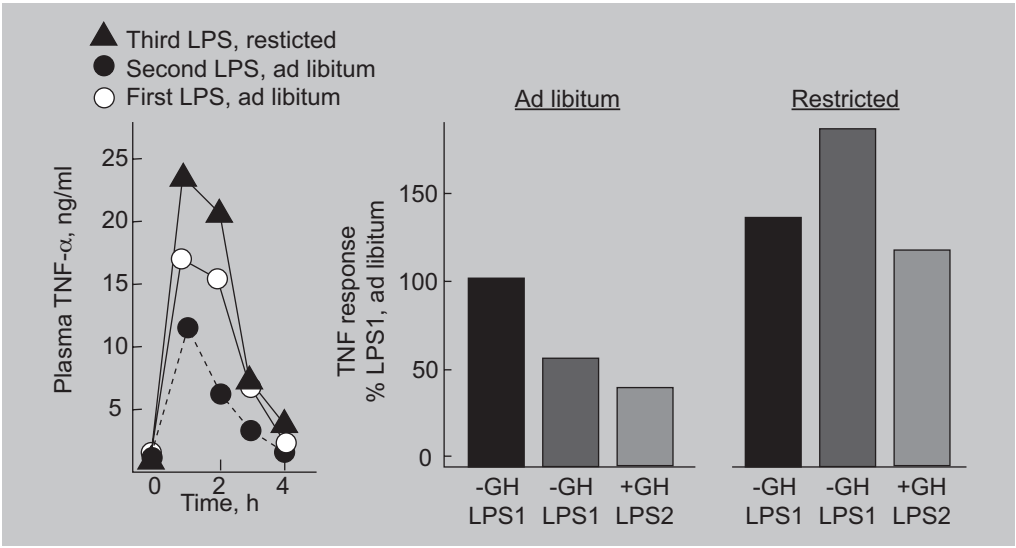

Figure 3. Reduced plane of nutrition "uncouples" the ability for animals to properly develop tolerance to repeated exposures to proinflammatory immune challenge, As modeled with repeated endotoxin challenge, peak plasma concentrations of TNF-α were properly suppressed after the second challenge with endotoxin (LPS, left panel). Upon restriction to maintenance intake, the TNF responses were augmented with accompanying increased perturbation of clinical signs. In the presence of short-term GH treatment (3-5 d, 100 µg/kg, sc; right panel) where TNF responses were blunted by both GH and repeated LPS challenge in ad libitum fed cattle, restruction resulted in an increased TNF response upon second LPS challenge in GH-treated cattle.

As seen in figure 3, when animals were fully fed and challenged with a low level of endotoxin (that minimally affected intake, if at all), following the switch to a calculated maintenance level of intake, cattle responded to the endotoxin challenge with increased levels of TNF-α and additional clinical signs of more severe reaction. Was this merely a coincidence that the intake levels that compromised IGF-1 function also perturbed the ability for animals to mount the downregulated proinflammatory survival response? Did the alterations in somatotropic axis underlie the perturbed TNF regulation? Research is continuing to construct the correct lines of parallelism in these nutrient-related observations.

Newer findings on the impact of GH on localized immune function/nitric oxide production

In considering the impact of endocrine mechanisms on immune function it has become necessary to consider how these interactions collectively modulate the target tissue response to immunological challenge. In particular, because many of the results of immune function are moderated through the refined production and fate of nitric oxide and superoxide, we decided to explore the effects of GH treatment on some specific aspects of nitric oxide production during a proinflammatory challenge as modeled with endotoxin administration. We used the model

presented in figure 4 to validate a process through which four critical control points (labeled as 1, 2, 3 and 4 in the figure) could be evaluated with regard to how GH might affect nitric oxide production. Figures 5 through 8 are representations of data published in Elsasser *et al*., 2004.

In figure 5, we acknowledge that the first needed event for NO production is entry of arginine into the cell (immunocyte or otherwise, critical control point 1). Arginine was identified as the *in vivo* substrate for any of the 4 isoforms of nitric oxide synthase (inducible, endothelial, neuronal and mitochondrial) where the guanadino N is cleaved to form NO (Soeters *et al*., 2002). Within the cell arginine is compartmentalized for metabolic activities largely related to urea cycle activity, NO production and protein synthesis. When activated by proinflammatory cytokines like TNF-α, specific cationic amino acid transporters are activated and facilitate the entry of arginine into the cell to support NO production. While GH appears to have little impact on the initiation of arginine transport into the cell via the CAT-2 transporter, GH does have a significant effect to limit the duration of activation and in essence work along homeostatic lines to prevent severe over production of NO which could further harm intracellular proteins (Figure 5).

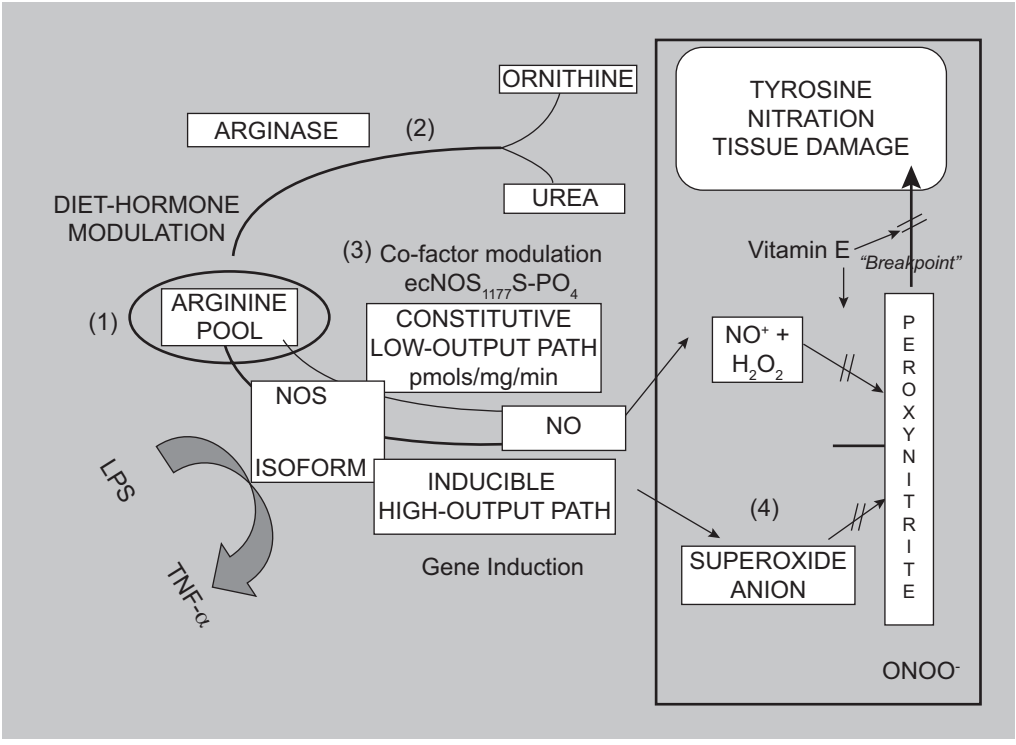

Figure 4. Critical control points (1 – 4) where growth hormone exerts significant effects on the enzymes and transport systems that regulate the production of nitric oxide from arginine under basal and proinflammatory challenge states (Source: adapted from Elsasser et al*., 2004).*

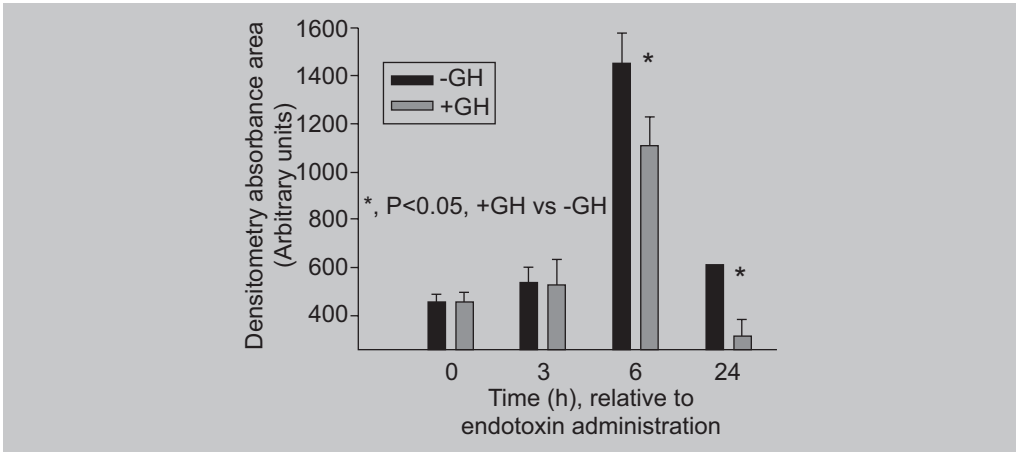

Figure 5. Relative expression of CAT-2 amino acid transporter mRNA as affected by endotoxin challenge (2.5 µg/kg, iv, E. coli 055:B5) and daily administration of recombinant bovine GH (0.1 mg/kg, subcutaneous). Data represent mean densitometry values for 5 steers per group normalized to a GAPDH housekeeping gene.

Within the cell, arginine availability to the nitric oxide cascade is in part determined by the prevailing activity of urea cycle enzymes (Hesse *et al*., 2001), type 2 arginase in particular (Wei *et al*., 2002). We had previously reported that GH treatment effectively reduced liver arginase activity (Elsasser *et al* 1996), an observation recently revalidated (Elsasser *et al* 2004, critical control point 2, Figure 6). Three interesting features of arginase activity that directly impact on how metabolic status can modulate nitric oxide activity are: (a) protein/energy relationships have

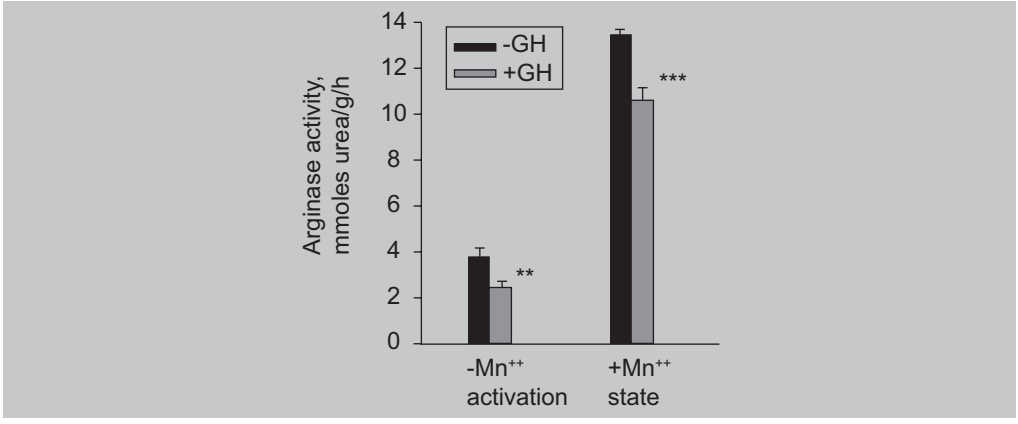

Figure 6. Relative basal and divalent ion-activated arginase activity as affected by daily administration of recombinant bovine GH (0.1 mg/kg, subcutaneous). Data represent mean values for urea generated from arginine from 10 steers per group. Data were from endotoxin-challenged animals. With no effect of endotoxin on the arginase activity, data from endotoxin-challenged and control, non-endotoxin groups were pooled thus generating the "n" of 10 steers/group.

opposite effects on arginase activity where, increases in protein intake increase activity while commensurate increases in energy with fixed protein intake reduce activity (Elsasser *et al*., 1996), (b) growth hormone administration suppresses arginase activity approximately 20 % in a time-dependant manner (treatment <3 days has no discernable effect; treatment >10 days decreased activity (Elsasser *et al*., 1996), and (c) the kinetic rate of arginine metabolism by arginase is more than one thousand-fold higher than the activity of nitric oxide synthase (μmoles/min/mg protein vs. pmoles/min/mg protein, Elsasser *et al*., 2004).

In addition, at the level of proinflammatory challenge used in these studies (*E. coli* 055:B5 endotoxin, 2.5 μg/kg, iv), the demonstrated increase in nitric oxide synthase activity was particular for the constitutive endothelial isoform Figure 7, critical control point 3), as modulated through an alternative signal transduction pathway for GH involving AKT/Protein Kinase B-enhanced activation of endothelial nitric oxide synthase (Figure 8) in the absence of a measurable increase in tissue nitric oxide synthase protein content (Elsasser *et al*., 2004).

The significance of the combined observations regarding the GH mediated simultaneous decrease in arginase activity and increase in endothelial NOS enzyme activity resides in the fact that arginase functions in the kinetic rate of μmoles substrate/min in contrast to pmoles substrate/min where a minor change in arginase activity results in a significant increase in the available arginine activity pools accessible by the nitric oxide cascade. Even though there is significant down regulation of the IGF-1 component of the somatotropic axis, an expression of GH regulation mediated via a down regulation of the JAK-2/STAT5 pathway, the AKT-driven phosphorylation activation of endothelial nitric oxide synthase (eNOS) was upregulated by GH treatment and endotoxin exposure without a change in eNOS protein content (Elsasser *et al*., 2004).

Finally, not only are the actions of NO affected by the rate of NO formation and effective concentrations generated, but the activity is also controlled by the metabolic fate of NO. A key to downregulating the activity of NO is the generation of superoxide anion during purine

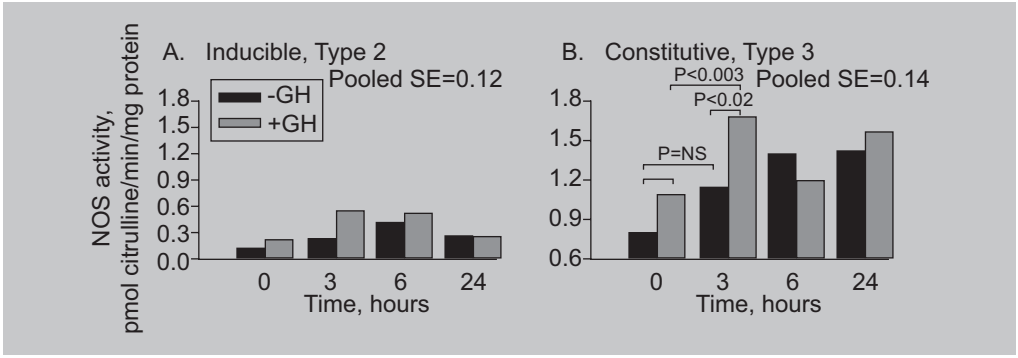

Figure 7. Effects of daily GH administration (0.1 mg/kg) and endotoxin challenge (E. coli 055:B5, 2.5 μg/kg, iv) on hepatic tissue enzyme activity of inducible (top panel) and constitutive endothelial nitric oxide synthase activity in steers. Data represent mean values for citrulline generated from radiolabelled arginine, n= 5 steers per group.

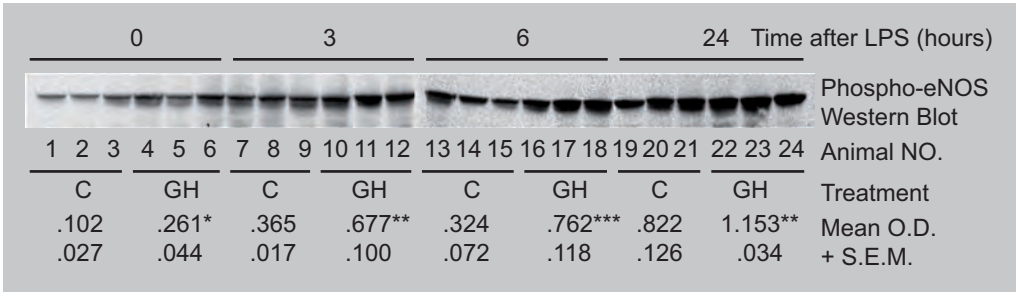

Figure 8. Daily administration of growth hormone (0.1 mg/kg, sc) for 1-12 d effectively increased both basal (time 0) and post-endotoxin phosphorylation activation of endothelial nitric oxide synthase in liver tissue of calves. This Akt-driven effect of GH was maintained or increased after endotoxin though there were JAK-STAT mediated decreases in IGF-1.

metabolism via xanthine oxidase (XO). Data from our laboratory indicate that GH also serves to upregulate XO activity (Figure 9, critical control point 4). A consequence of the interaction of NO with superoxide anion is the generation of powerful nitrating reactants such as peroxynitrite (Ischiropoulos, 2003; Gow *et al*., 2004), a reactant that forms nitrotyrosine in tyrosine-containing proteins. Overproduction of nitrotyrosine and nitrated proteins has been associated with the loss of function of key metabolic and signal transduction enzymes due to spatial and charge hindrance of major reaction site epitopes. Thus in the liver, where we have evidenced a significant increase in protein nitration following endotoxin challenge in GH-treated animals (Figure 10), the presence of nitration may represent a part of the needed signaling to redirect metabolism

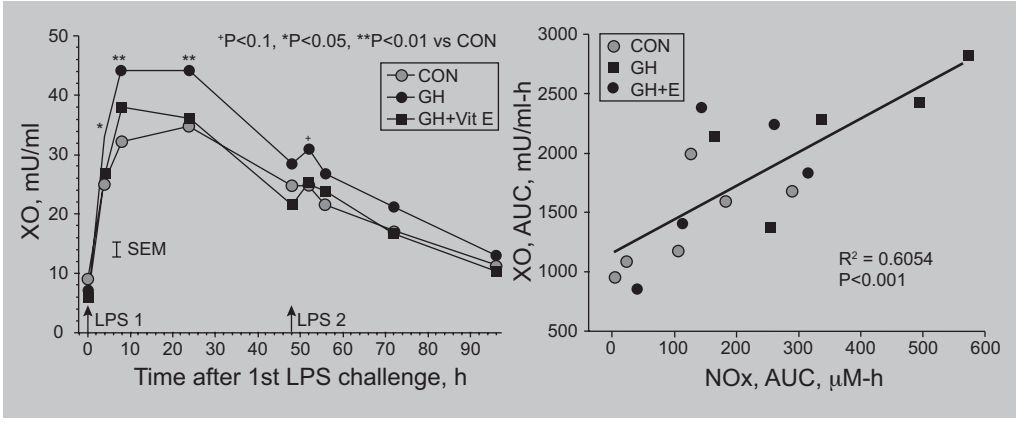

Figure 9. As the source for much of the superoxide anion needed to generate the nitrating reactant peroxynitrite, xanthing oxidase activity inceases with endotocin challenge and is further augmented in GH-treated animals. (Left panel) Effects of daily GH injections (0.1 mg/kg, subcutaneous) on changes in plasma xanthine oxidase activity following challenge of steers with E. coli *055:B5 endotoxin. Two endotoxin challenges, 2.5 µg/kg, iv, 5 days apart were administered to steers, n=5/group.(Right panel). Xanthine oxidase activity is highly correlated with the generation of NO from arginine (From Kahl and Elsasser, 2004).*

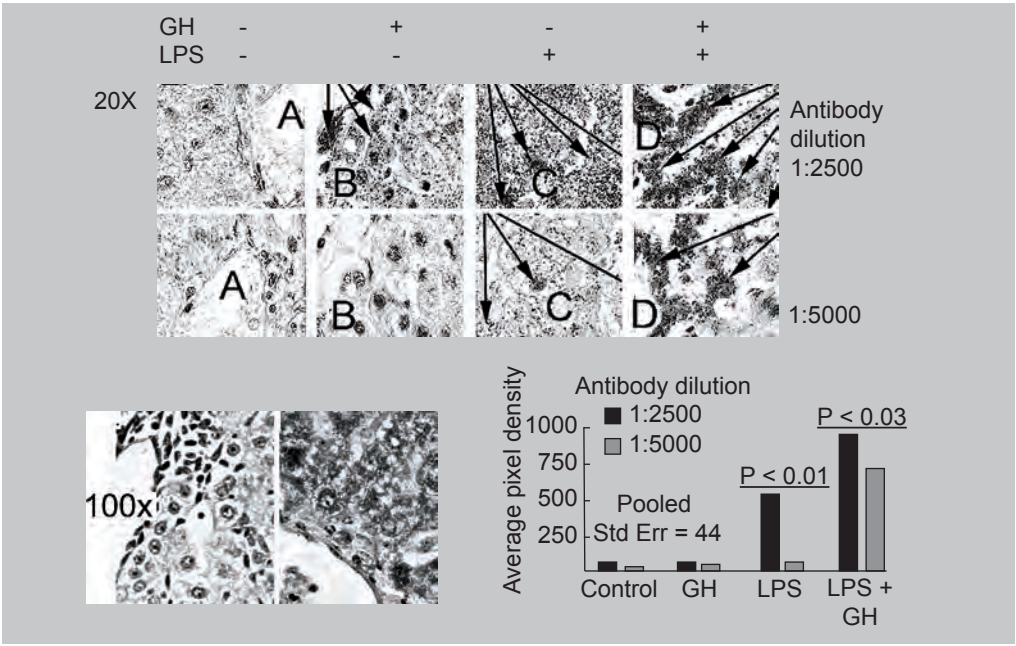

Figure 10. Immunohistochemical localization patterns derived using anti-nitrotyrosine antibody, demonstrate the absence and significant increased presence of hepatic liver protein nitration in tissue from steers challenged with E. coli endotoxin (serotype 055:B5, 2.5 µg/kg, iv) and its up-regulation in the presence of growth hormone (GH, Top Panel). Letters "A" through "D" indicate common elements of tissue architecture to serve as localization reference points for the serial tissue sections used at the two different antibody concentrations. Digital image analysis and quantification of color spectrum-specific specific pixels derived from the horseradish peroxidase-DAB reaction were applied to specimens stained at two dilutions of antibody to further discriminate antigen abundance on the basis of sensitivity of immunostaining. (Bottom Right Panel). High magnification (Bottom Left Panel) shows the cytoplasmic increased intensity of the punctuate antigen localization.

away from synthesis/anabolism (other than increased synthesis of acute phase response proteins) and towards nutrient sparing for repair and immune function priorities. Collectively, these data indicate that many of the actions of growth hormone to shape the character of the immune response are manifest at both the level of the immune reactant itself (ie., cytokine and prostaglandin response to proinflammatory stimuli) as well as at the cellular target level where both endocrine and immune signals are integrated to shape the response of tissues.

When all of the participating factors converge to generate an aberrant oxidative proinflammatory response, we observe consistent correlates in the development of liver protein nitration and the propensity for that nitration to underlie much of the disturbance in IGF-1 regulation (Figure 11). The interesting feature of this observation is that the degree of nitration can be modulated if, in anticipation of the proinflammatory stress challenge, the antioxidant character of cells is fortified through direct injection of mixed isomer tocopherol, vitamin E. In figure 9, when 1200

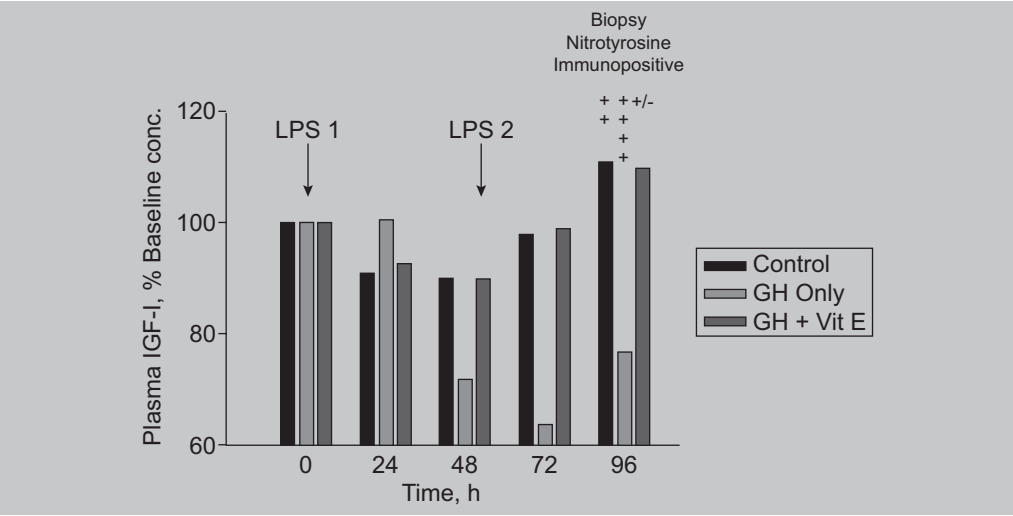

Figure 11. The effects of liver protein nitration 96 h after endotoxin challenge to steers are correlated with the measured decreases in circulating plasma concentrations of IGF-1 in control and GH-treated steers. Not only could GH treatment not maintain the higher levels of IGF-1 present in GH-treated steers immediately

IU of vitamin E was injected im for 5 days prior to dual endotoxin challenge, plasma IGF-1 levels were minimally perturbed as were aspects of feed intake and general weight loss (Elsasser *et al.*, 2000).

When we entertain the presence of the target tissues in the conceptualization of how the endocrine system impact immune function, we are further drawn towards the realization that what we are dealing with is a dynamic restructuring of tissue priorities, where especially during this critical periparturient time, the localized paracrine interactions become so significant, albeit difficult to quantify. Most prominent in these reprioritizations of tissue needs are situations where (a) during nutrient excess and homeostatic stability adipose tissues become high priority nutrient suppliers under the proinflammatory signals that stimulate lipoprotein lipase and down regulate fat synthesis, (b) lymphoid and hepatic cells shift to high priority metabolism in terms of white blood cell trafficking and tissue infiltration and the generation of acute phase response proteins, respectively, and (C) the abrupt switch to colostrum and mammary antibody synthesis and subsequent milk production, all occurring with a measured degree of nutrient intake compromise.

The endocrine - immune gradient and integration of priority signals

As a start towards understanding how these priorities change between tissues or with respect to, for example, the shift in priorities for nutrient use from growth in the young animal to reproduction and further to lactation in the older animal, it is useful to employ a framework model that depicts a relationship between tissue and biological function priorities. Sir John Hammond constructed a logic paradigm based on metabolic need and drive of tissues (Figure

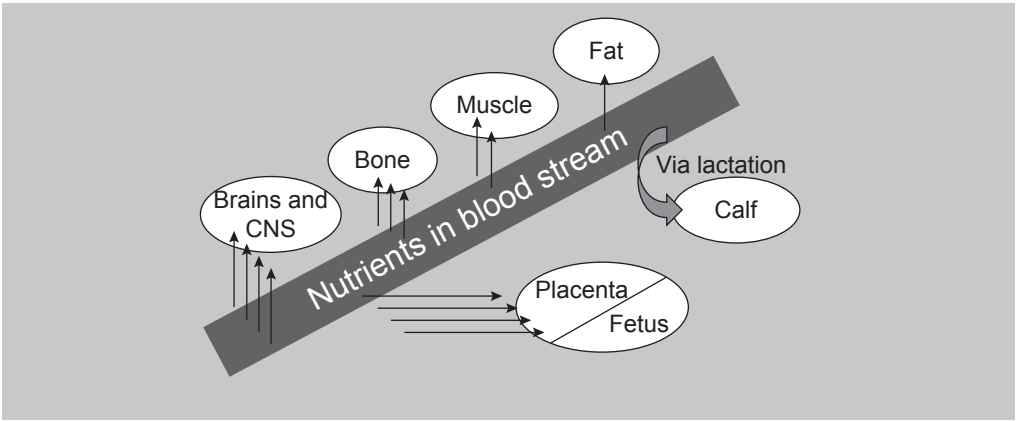

Figure 12. As adapted from Hammond (Hammond, 1952), a hierarchy of priorities exists between tissues wherein available nutrients are partitioned and distributed based on a functional metabolic need as well as concentration in the blood. In what can be envisioned as a separate "compartment", the fetal-placental unit commands a high percentage of metabolic resources prior to birth, as does the initiation of lactation to support the calf postpartum.

12) as appreciated and understood in 1952 (Hammond, 1952) where neural function and in the female, fetal-placental-lactation development, were set at a high priority, in contrast to adipose tissue priorities, especially when dietary energy supply was limited. We expanded upon and updated his basic theme by superimposing upon metabolic priority a general set of regulatory effectors termed the "endocrine-immune gradient" (Elsasser *et al*., 2000) wherein tissue needs were orchestrated between tissue types, (ie., adipose, muscle, visceral organ) via hormone (endocrine) and cytokine (immune) messengers. With more recent information (readers are directed to the excellent summarizations of nutrient partitioning within the fetal-maternal-placental unit, Bell and Ehrhardt, 2000, as well as the homeostatic and homeorhetic controls present in the rearrangement of nutrient use in lactating cows, Bauman, 2000) we can now evolve this model (Figure 13) to express a subprioritization within tissues where, for example, postural and locomotor muscles differ in fiber type make-up (Elsasser *et al*., 2003) or the selective maintenance of fatty tissues adjacent to lymphoid structures (Pond, 2003). This area of localized paracrine effector function is changing so rapidly with the discovery of the capacity for previously unidentified tissues to be sources for so many peptides with hormone and cytokine character, that the actual definition of an "endocrine tissue" is changing. Where previously relegated to a rather nonglamorous position of low metabolic priority, adipose tissue is now recognized as a key feature in the complex regulation of endocrine-immune signaling because of its ability to synthesize and release significant quantities of leptin (Ingvartsen and Andersen, 2000; Ingvartsen and Boisclair, 2001). Adipose tissue potentially respond to immunological stimuli directly via the adipocyte membrane based endotoxin receptor CD-14 (Daniel *et al*., 2003) as well as secrete different molecular forms of TNF-α (Coppack 2001) that are purported to contribute significantly to glucose homeostasis via TNF-directed peripheral (muscle) insulin resistance (Smith *et al*., 1999).

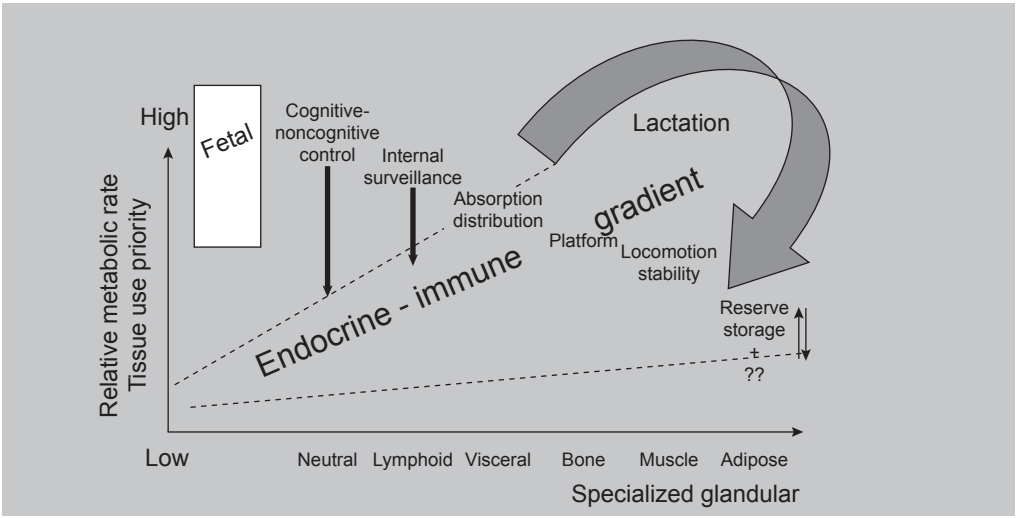

Figure 13. Adding to the Hammond model, the importance of visceral tissue, lymphoid tissues and a more dynamic relationship in the metabolic priority for nutrient partitioning are governed in endocrine-immune system interactions. The large arrow emphasizes the ability for the endocrine-immune gradient, with blood-borne as well local paracrine–elaborated factors to rapidly change a tissue's metabolic priority from low to high, as when stress challenges dictate a needed localized release of energy substrates from adipose to adjacent immune cells. Smaller shaded arrows indicate the pituitary origin (neural-CNS) for so many of the endocrine responses to stress and lymphoid cell-derived (fixed, circulating and infiltrating) regulatory cytokines. Specialized glandular functions like lactation need a high priority "compartment" that has its own challenges as regards the need to make large quantities of milk all the while fending off pathogen insults that trigger mastitis.

In the updated rendition briefly reviewed here, additional literature resources have allowed the tissue hierarchy to be presented as a more fluid and dynamic collective. This is particularly true and evident in the periparturient cow where immunological stressors that activate proinflammatory-type response cascades dictate a necessary "hold" on many anabolic functions in the dam while proportionately channeling the needed nutrients to the developing fetus, or immune tissues needed to fight the insult, or the mammary gland for milk. In fact the processes surrounding parturition itself have been suggested to represent a proinflammatory state in cows (Koets *et al*., 1998), as is the proinflammatory cytokine-driven tissue remodeling needed to facilitate the release of the fetus from the placenta and dilation of the cervix (Fujimoto *et al*., 2002).

A good example of this localized reordered priority can be seen in recent insights to the functionality of different adipose storage depots. Not all fatty tissues can be regarded simply as "structures of excess nutrients and storage". Recent data indicate that the adipose deposits surrounding lymphoid structures may, in fact, retain a position of relative high nutrient partitioning priority to specifically serve as a fuel depot to support specific immune function metabolism during periods of prolonged disease and accompanying anorexia (Mattacks *et al*., 2003; Pond, 2003). In addition, we notice that there is not only a relatively variable impact of the endocrine immune gradient

on different tissues but a temporal relationship as well. What must be remembered however is the fact that even the present status of data reflecting the endocrine regulation of immune function may be grossly underrepresented by inferences made through traditional measurements of plasma and even levels of extracted hormones and cytokines, as protein or mRNA. Pond (2003) clearly demonstrates the paracrine influence of activated lymphoid tissue on the immediately adjacent adipose tissues where the needs of the immune cells direct the lipolytic activities of the adjacent fat cells. The ultimate reflection of endocrine and immune interactions may evolve to be best viewed in terms of the signal transduction processes that further direct protein function.

Relatively new to the tissue priority/nutrient partitioning scheme is the addition of the immune system structures. Certainly critical in regard to species survival, the lymphoid tissues comprising the immune system have been rather difficult to position in the relative scheme as defined by metabolic criteria. This was mainly due to the fact that until recently, there have been relatively few documented estimations of the actual size of the immune system and under what terms (physiological states) the metabolism of the participating cells is studied. In this regard, where the physical mass of the tissues compromising the immune system may account for less than three to four percent of available nutrient use in times of health a sizeable increase in metabolic demand during stress is induced, at least as approximated by lysine demand (Klasing and Calvert, 2000). Similar numerical values were deduced from investigational literature sources by Kehrli *et al*., (2005) where the presentation of a perceived immune stress disproportionately increases the immune demand for nutrients even when perturbed feed intake and gut absorption may challenge the generalized cellular availability (Demas *et al* 1997). This increased demand numerically does not even take into account the added caloric requirements of adjunct tissues recruited to generate the thermal fever response, previously estimated at more than a 30% increase in metabolic rate for each 1.5 degree centigrade increase in core temperature (Baracos *et al*., 1987; reviewed in Elsasser *et al*., 2000). Thus in terms of maintaining a high priority biological function such as fetal development and lactation when nutrients and calories are limited, some tissues may need to be called on to supply the energy deficit and so the endocrine and immune hormones that regulate metabolism take on additional challenges that directly or otherwise affect the metabolism of tissues.

The activation of the proinflammatory immune response changes many of the tissue priorities when catabolic actions are called upon to provide needed energy and amino acid reserves for acute phase protein synthesis, thermogenesis, and maintenance of homeostatic equilibrium (Elsasser *et al*., 2000). The pituitary serves as a direct link between the higher priority neural center and many of the effects of the immune system through modulatory effects of the adrenal glucocorticoids, reproductive steroids, as well as somatotropic (growth hormone and insulin-like growth factor-1) and lactogenic (prolactin, placental lactogen) axis hormones. In fact, the pituitary serves as a direct target for the actions of several immune cytokines, possessing not only specific cytokine receptors that are integral to the sensitivity and responsiveness of pituicytes to hypothalamic regulatory releasing and inhibiting peptides (Elsasser *et al*., 1991; Abraham, *et al*., 1998). To a large extent, therefore, when one is addressing the influence of the endocrine system on immune function, we are faced with the realization that the relative background health and metabolic status determine how a hormone might affect a given immune function. This regulatory capacity may not (a) be consistent between periods of health and stress, (b) not be readily characterizable

in terms of peripheral hormone patterns, and (c) be over-ridden by physiological demands of other issues such as parturition and even animal management philosophy. As the sensitivity of methods of detection are refined, we are recognizing that many of these regulatory endocrine and immune effectors are produced in far more tissues than previously identified, though their functions in these domains remain to be elucidated.

The greatest complication to modeling endocrine effects on immune system function lies in the fact that only at the level of the target cell can the outcome of all endocrine and immune effector interactions be appreciated. It is only through cellular integrated signal transduction processes that a cell's function changes, the change reflecting the localized "priority" of that space and at that place in time. An extensive literature database exists wherein effects of endocrine hormones are characterized for their impact on immune processes. Generalizations may pertain to the cow, but for the most part many of the so-called documented effects of endocrine secretions on immune function are not only somewhat species-specific but in many ways specific to the dairy cow during the interval surrounding parturition.

The swing between anabolic and catabolic processes created through the immunosurveillance and response to disease vectors was characterized in terms of the evolved proinflammatory cytokine cascade (i.e., tumor necrosis factor-α down through nitric oxide generation) to redirect metabolic priorities for the purpose of directing nutrient availability and use for the so-called processes of higher life function. Thus what we see is, in effect, hormone-cytokine-tissue regulatory loops within loops that establish the fine tuning of hormone directed immune function and impact on tissue performance.

Adrenomedullin – a novel bridge in the endocrine regulation of immune system function

In characterizing the nature of the relationships present in the endocrine input to immune system function, it becomes apparent that the presentation of the endocrine signal to the responding immune cell is multidimensional with far more subtle aspects of regulation evolving from the patterns of hormones that reach the target cells than simply the changing concentration. The complexity of the interaction is intensified for the immune system components perhaps more so than other physiological systems. The immune system is a "moving target" changing functional locations continuously within the gradient between health and disease. As such, the effects of a hormone on an immune response will be modulated according to how the prevailing systemic and localized situation is being internally interpreted and reacted to. Similarly, as a tissue responds and adjusts with countermeasures to the prevailing signals, one sees the impact of a multitude of physiological processes that affect how target cells immune cells receive endocrine signals.

Adrenomedullin (AM) is a 52 amino acid peptide discovered by Kitamura and colleagues (Kitamura *et al*., 1993) in an acidic chromatographic fraction from an adrenal pheochromocytoma extract. Initially characterized as a regulator of vascular tone, AM is now recognized as a major component of the calcitonin/calcitonin gene- related peptides (CGRP), credited with regulatory activities throughout the body linking physiological systems through its integration into many of the core biochemical processes. With the development of antibodies and nucleic acid probes, not

only has the localization of AM and its different receptors throughout the body been documented, but it is now realized that AM is phylogenetically "old" as a regulator of cell processes, its presence being detected throughout vertebrate and down to some unicellular and invertebrate species (Clark *et al*., 1998; Martinez, *et al*., 1996). This ancient functionality partners well with what is now also recognized as one of the major means by which AM affects cell function, the regulation and metabolic fate of nitric oxide, itself a phylogenetically ancient signaling and protective molecule. This ability of AM to integrate tightly with the nitric oxide synthase cascade lies at the heart of its biological actions that include regulation of vascular tone (Minamino *et al*., 2000), cardiovascular homeostasis (Shimosawa *et al*., 2003), regulation of pancreatic hormone secretion (Zudaire *et al*., 2003) and hormone activity (Martínez et al., 1998a, multiple effects on the inflammatory process (Elsasser and Kahl, 2002, gastrointestinal secretion and motility (Martínez *et al*., 1998b), electrolyte homeostasis (Taylor and Samson, 2002), and deeply involved with the complex autocrine regulation of tumor growth, metastasis, and angiogenesis (Cuttitta *et al*., 2002). Particularly relevant to the discussion at hand, additional actions of AM involve preservation of cell function through its anti-apoptotic actions in the immune and cardiovascular systems (Belloni *et al*., 2003; Yin *et al*., 2004), modulation of cluster differentiation protein CD-11b on neutrophils (Saito *et al*., 2001**),** production by macrophages via bacterial toxins (Zaks-Zilberman *et al*., 1998**)**, its localization in the mammary gland (Welsch *et al*., 2002; Jahnke *et al*., 1997), its secretion into human and bovine milk (Pio *et al*., 2000), and the unique character to function as an antimicrobial peptide in skin, oral and bronchial epithelia and gut mucosa (Allaker *et al*., 1999) as well as in amniotic fluid and fetal membranes (Macri *et al*., 1996). Its presence in milk complements the myriad of bioactive peptides present in milk (Clare and Swaisgood, 2000; Van Hooijdonk, *et al*., 2000).

We recently reported for the first time the presence of a specific binding protein for AM in the plasma of multiple species and the variability of concentration of the binding protein between states of health and disease (Elsasser *et al*., 1999). Subsequent to this observation, we further characterized the protein as a unique regulatory component of the immune complement cascade, complement factor H (Pio *et al*., 2001). Factor H is the key regulatory component in the Factor I-dependant progression of cascade cleavage of complement C3b. When AM binds to this regulatory Factor H molecule the rate of C3b split is augmented. Two domains capable of binding AM in the Factor H molecule were tracked to high and low affinity regions, respectively (Martínez et al., 2003). Specific function characteristics of the high and low affinity sires are yet to be explored but suggest the capacity to tailor drug developments for intervention strategy purposes to these regions.

A reciprocal level of interactions is imparted on biological properties of AM when it is bound to the Factor-H binding protein. We demonstrated that the AM antimicrobial peptide activity against *E. coli* was significantly reduced when AM was linked to its binding protein. Using standard tests of antimicrobial activity based on the conventional minimal inhibitory concentration, Factor H shifted the minimal inhibitory antibiotic concentration (MIC) from ~10 to >35 μg/ml (Pio *et al*., 2001). Collectively, therefore we can envision a significant role for this endocrine regulator to affect and regulate immune function through effects on immune nitric oxide regulation, survival of immune tissues, expression of immunoregulatory antigenic determinates, and the overall health of tissues as impacted by infectious agents, oxygen supply, waste elimination, hormone-directed nutrient availability to tissues (pancreatic insulin and glucagons regulation), and complex signal

transduction integration functioning through the phosphatidal inositol/AKT pathway (Miyashita *et al*., 2003), a commonly shared link in the signal transduction activity through which TNF-α regulates endothelial nitric oxide synthase via phosphorylation of the serine at position 1177 (Kawanaka, *et al*., 2002).

The myriad of influences on cell response to a biosignal is reviewed in Table 2. Thus, adrenomedullin is capable of affecting tissue responses to itself as well as other hormones and cytokines by direct effects (through its own receptors) or indirect effects (altering vasopressor activity and arterial perfusion kinetics supplying oxygen and hormone-directed nutrient supply), as presented in Table 2.

As relates to the periparturient cow and its varying levels of immunocompetance, we asked whether tissue-specific changes in AM could be localized to the mammary gland. That could provide an initial clue as whether a part of the problem might relate to poor activity of AM. Using both in situ hybridization and sensitive quantitative immunohistochemical techniques we (Elsasser *et al*., 2004) discovered that there was a significant change in the mammary alveolar content of AM as well as the Factor-H/AM binding protein. Comparing tissues obtained from cows in different stages of lactation, we observed that the lowest level of expressed AM in the mammary gland occurred in tissue sections obtained from cows in the dry period and close after calving (Figure 14). The importance of these observations relate to the inherent capacity for the mammary gland to mount defenses against potential invading microorganisms that could trigger clinical mastitis including the local production of inflammatory cytokines (Schmitz *et al*., 2004) generation of superoxide anion via mammary xanthine oxidase (Bruder *et al*., 1982, Collins *et al*., 1988) and nitric oxide (Komine *et al*., 2004). First, the antimicrobial peptide capacity of AM is lowest at the time when it is most needed. This is also the case for lactoferrin and other endogenous peptides

Table 2. Ten influences that impact tissue responses to the fluctuating hormone-cytokine milieu (adapted from Elsasser et al. *2000).*

1.	Changing concentrations of hormones and cytokines that reach cells from remote endocrine, paracrine and autocrine modes of effector presentation
2.	Changing temporal character of how these concentration changes are presented to the cells (concentration x blood flow = flux)
3.	The presence of receptors for the effector ligands on and within cells
4.	Blood flow, nutrient and oxygen presentation/CO_2 removal to and from tissues
5.	Alterations in intracellular signal transduction interactions (cAMP, Ca^{++}, kinase phosphorylation activation, phosphodiesterase activity, RMP proteins, etc.)
6.	Plasma transport binding protein modulation of cell effector availability
7.	Trans-endothelial permeability
8.	Mitochondrial density in tissues and energetic functionality
9.	Rates of effector degradation and metabolic clearance
10.	Regulation of gene transcription, and translation, ribosome stability, intracellular cytoskeletal-dependant resource shuttling, nutrient-specific gene and regulatory elements/ cell cycle-dependant responses

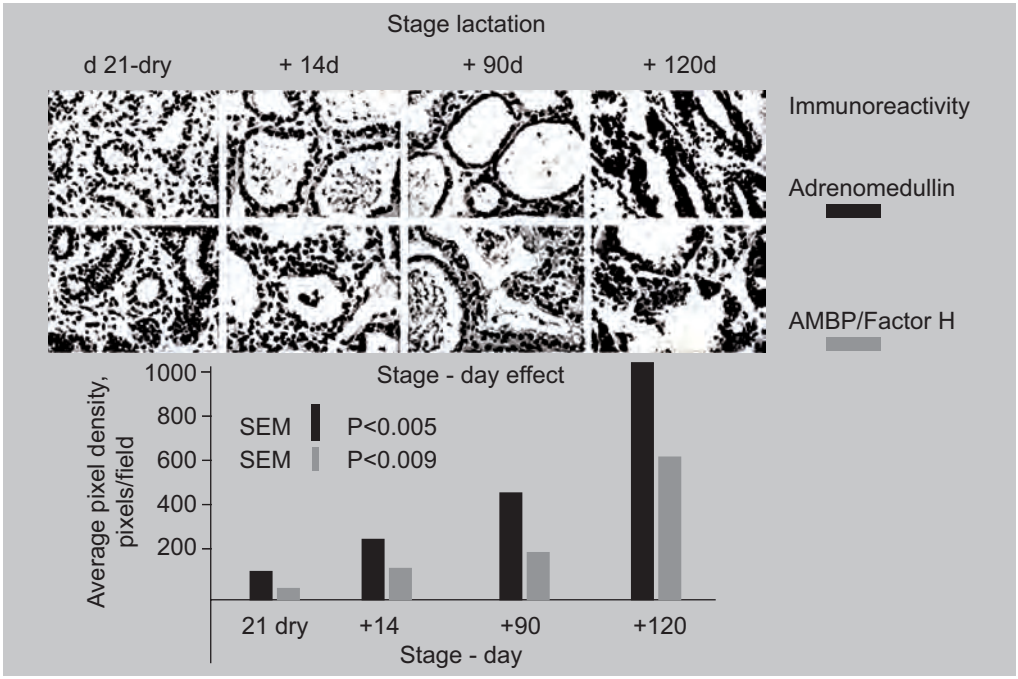

Figure 14. Mammary tissue presentation of adrenomedullin and its binding protein (AMBP/Factor-H) vary throughout lactation. Lowest in the dry period before parturition, through 14 days into lactation, mammary content of these multifunctional molecules appears to parallel the relative degree of compromised and resolved immune function.

in milk that, as a collective milieu, form one of the basic compartmentalized local mediators of mammary tissue health, not unlike that present in epidermal skin, the respiratory tract, and even the GI tract (Allaker *et al.*, 1999). In this regard, we can also envision a potential compromise of the nitric oxide and superoxide anion defense mechanisms since these are also lowest at this time period. In addition, the tight linkage in regulation of AM and nitric oxide make a strong link for blood flow regulation in the mammary gland to be regulated by the local production of these vascular mediators (Lacasse *et al.*, 1996).

Several lines of evidence suggest that the local tissue growth factor composition, IGF-1 (Dasu *et al.*, 2003) and adrenomedullin (Miyashita *et al.*, 2003; Hashimoto *et al.*, 2000) in particular, affects significantly the capacity of tissues to repair themselves following injury. We have explored a functional link in the relationship between adrenomedullin expression in the liver and the capacity for cattle to resist the drop in plasma IGF-1 observed with proinflammatory challenge with bacterial endotoxin. Where cattle were challenged with endotoxin (Elsasser *et al.*, 2002), based on the magnitude and duration of the elevated plasma and liver adrenomedullin peptide responses (RIA and quantitative immunohistochemistry), we were able to clearly stratify animals into two distinct populations – high AM responders and low AM responders. This reminds us again of the response stratification observed in dairy cattle by Mallard (1997) in experiments conducted

at the University of Guelph in Canada and the variability in immune response observed there had specific endocrine ramifications associated with it. Adrenomedullin is normally in rather low abundance in the liver, in contrast to other tissues such as the lung and pancreas where it is constitutively present and a significant component of defense and hormone regulatory systems. Upon proinflammatory stimulation AM increases in the liver, similar to the levels of stimulation observed with taxol or endotoxin stimulation of macrophages (Zaks-Zilberman *et al.*, 1998). Interestingly, the cattle where the AM levels increased and remained elevated were the ones in whom the pro-oxidative stress of endotoxin challenge had the lowest impact on alterations in IGF-1 (Figure 15). As can be observed in the final panel on the right side of Figure 15, there is a linear and positive correlation between the level of AM response and the capacity to maintain apparent better control (author's subjective interpretation) of IGF-1 than that suggested by the measurements of low IGF-1 levels in the poor AM response animals. We are presently pursuing whether the upregulation of AM in the higher IGF-1 level animals represents an ability to utilize some of the antiapoptotic, increased perfusion and nitric oxide regulatory attributes of AM. If this is the case, certainly the potential to further modulate AM responses in the mammary gland or within the periparturient cow in general might merit further examination.

Conclusions

Animals will experience certain natural levels of stress in their lives, beginning with birth – for the cow as well as the calf. The periparturient cow has been characterized as immunocompromized and this state is credited with the potential for this animal to be more susceptible to stress and infections disease. There is variability throughout cattle populations in the extent to which this

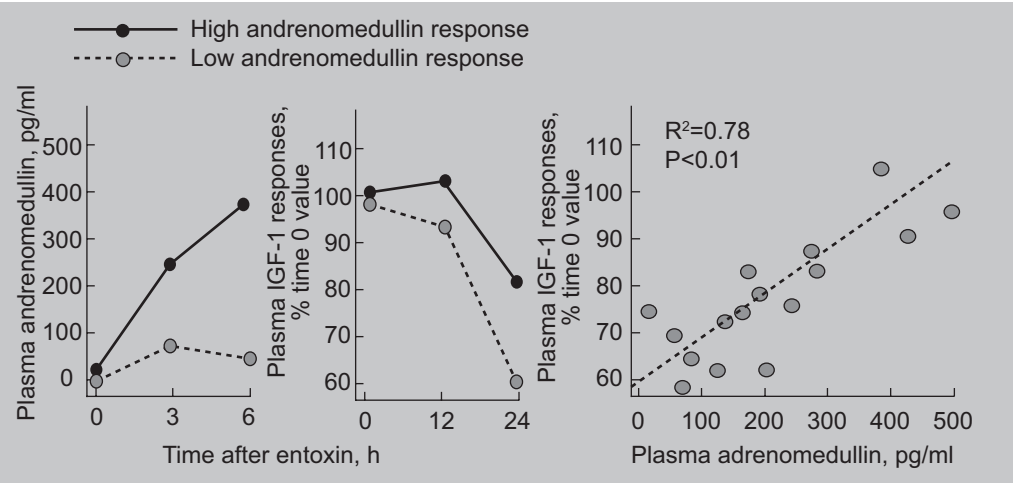

Figure 15. Differences in the population variability in capacity to generate tissue preservation responses such as increased adrenomedullin expression during proinflammatory stress may be a key factor affecting the general health of animals. Cattle responding to endotoxin challenge with high levels of adrenomedullin preserved other functions such as regulation of IGF-1 (left, middle panels). The degree to which AM was increased was directly correlated with the level of IGF-1 (right panel).

immunocompromised state is clinically presented and there are several endocrine imbalances that may impact how this deficiency develops. Management practices that push animals to mobilize and redirect tissue nutrient stores appear to add to the basic physiological state through which the mother has made adaptations in her immunosurveillance to permit conception, implantation and pregnancy to follow to term. The progesterone-estrogen-prostaglandin axis contributes significantly to this immunomodulation. The somatotropic axis components, GH, IGF-1 and respective signal transduction and binding protein modulators, are reflective of the basic underlying metabolic status of the term animal and may function as the metabolic interpreters of immune function, certainly in as far as changing physiological tissue priorities need to change to permit the survival of the offspring. In this regard new information on relationships between the immune system and adipose tissue suggest that endocrine, and in particular paracrine, effectors released from fat cells (ie., leptin and TNF-α) may significantly shape the reactiveness of specific immune functions. Finally, multifunctional hormones such as adrenomedullin may be shown to play a role in endocrine modulation of immune function where especially in the periparturient cow, opportunities may exist to boost the capacity for tissues to be more resilient and refractory to many of the adverse effects of endogenously and overly produced immune mediators such as nitric oxide, superoxide anion and various peroxides.

References

Abraham, E.J., J.N. Morris-Hardeman, L.M. Swenson, E.L. Knoppel, B. Ramanathan, K.J. Wright, D.M. Grieger & J.E. Minton (1998). Pituitary function in the acute phase response in domestic farm animals: cytokines, prostaglandins, and secretion of ACTH. Domestic Animal Endocrinology, 15(5), 389-396.

Adams, N.R., J.R. Briegel, M.J. Thompson & L.M. Sammels (2000). Metabolic hormonesand tissue concentrations of mRNA for IGF-I in lines of sheep that differ in their protein synthesis response to feed intake. Journal of Endocrinology, 167(2), 315-320.

Allaker, R.P., C. Zihni & S. Kapas (1999). An investigation into the antimicrobial effects ofadrenomedullin on members of the skin, oral, respiratory tract and gut microflora. FEMS Immunology and Medical Microbiology, 23, 289-293.

Baracos, V.E., W.T. Whitmore & R. Gale (1987). The metabolic cost of fever. Canadian Journal of Physiology and Pharmacology, 65(6), 1248-1254.

Bauman, D.E. (2000). Regulation of nutrient partitioning during lactation: Homeostasis and homeorhesis revisited. In: P.B. Cronjé, (ed.) Ruminant Physiology; Digestion, Metabolism, Growth and Reproduction. CABI Publishing, New York, pp. 311-328.

Bazer, F.W. & N.L. First (1983). Pregnancy and parturition. Journal of Animal Science, 57 (Suppl 2), 425-460.

Bell, A.W. & R.A. Ehrhardt (2000). Regulation of macronutrient partitioning between maternal and conceptus tissues in the pregnant ruminant. In; P.B. Cronjé, (ed.) Ruminant Physiology; Digestion, Metabolism, Growth and Reproduction. CABI Publishing, New York, pp. 275-293.

Belloni, A.S., M. Trejter, L.K. Malendowicz & G.G. Nussdorfer (2003). Adrenomedullin stimulatesproliferation and inhibits apoptosis of immature rat thymocytes cultured in vitro. Peptides, 24, 295-300.

Boos, A., J. Kohtes, A. Stelljes, H. Zerbe & H.H. Thole (2000). Immunohistochemical assessment of progesterone, oestrogen and glucocorticoid receptors in bovine placentomes during pregnancy, induced parturition, and after birth with or without retention of fetal membranes. Journal of Reproduction and Fertility, 120(2), 351-360.

Burton, J.L., S.A. Madsen, J. Yao, S.S. Sipkovsky & P.M. Coussens (2001). An immunogenomics approach to understanding periparturient immunosuppression and mastitis susceptibility in dairy cows. Acta Veterinary Scandinavia, 42, 407-424.

Burton, J.L., T.H. Elsasser, T.S. Rumsey, J.K. Lunney & B.W. McBride (1993). Immune responses of growing beef steers treated with estrogen/progesterone implants or insulin injections. Domestic Animal Endocrinology, 10(1), 31-44.

Bruder, G., H. Heid, E.D. Jarasch, T.W. Keenan & I.H. Mather (1982). Characteristics of membrane-bound and soluble forms of xanthine oxidase from milk and endothelial cells of capillaries. Biochemica and Biophysica Acta, 701, 357-369.

Burgess, W., Q. Liu, J. Zhao, Q. Tang, A. Ozawa, R. VanHoy, S. Arkins, R. Dantzer & K.W. Kelley (1999). The immune-endocrine loop during ageing: role of growth hormone and insulin-like growth factor-I. Neuroimmunomodulation, 6, 56-68.

Burvenich, C., E. Monfardini, J. Mehrzad, A.V. Capuco & M.J. Paape (2004). Role of neutrophil polymorphonuclear leukocytes during bovine coliform mastitis: physiology or pathology? Verh K Acad Geneeskd Belg. 66(2), 97-150.

Capuco, A.V., D.L. Wood, T.H. Elsasser, S. Kahl, R.A. Erdman, C.P. Van Tassell, A. Lefcourt & L.S. Piperova (2001). Effect of somatotropin on thyroid hormones and cytokines in lactating dairy cows during ad libitum and restricted feed intake. Journal of Dairy Science, 84, 2430-2439.

Challis, J.R., D. Sloboda, S.G. Matthews, A. Holloway, N. Alfaidy, F.A. Patel, W. Whittle, M. Fraser, T.J. Moss & J. Newnham (2001). The fetal placental hypothalamic-pituitary-adrenal (HPA) axis, parturition and post natal health. Mol Cell Endocrinol. 20;185(1-2):135-44.

Chaouat, G. (1987). Placental immunoregulatory factors. J Reprod Immunol. 10(3):179-88.

Clare, D.A. & H.E. Swaisgood (2000). Bioactive peptides in milk: a prospectus. Journal of Dairy Science, 83, 1187-1195.

Clark, A.J.L. & S. Lowe (1998). Adrenomedullin receptors and their genes: A family in evolution. In: Adrenomedullin. Martínez, A. & Cuttitta, F., eds. IOS Press, Washington, DC, pp 41-48.

Cole, N.A. & F.S. Guillot (1987). Influence of *Psoroptes ovis* on the energy metabolism of heifer calves. Veterinary Parasitology, 23, 285-295.

Collins, R.A., K.R. Paarsons, T.R. Field & A.J. Bramley (1988). Histochemical localization of xanthine oxidase in the bovine mammary gland. Journal of Dairy Research, 55, 25-32.

Coppack, S.W. (2001). Pro-inflammatory cytokines and adipose tissue. Proceedings of the Nutrition Society, 60(3), 349-356.

Cuttitta, F., R. Pio, M. Garayoa, E. Zudaire, M. Julian, T.H. Elsasser, L.M. Montuenga & A. Martínez (2002). Adrenomedullin functions as an important tumor survival factor in human carcinogenesis. Microscopy Research and Technique. 57(2):110-9.

Daniel, .JA., T.H. Elsasser, C.D. Morrison, D.H. Keisler, B.K. Whitlock, B. Steele, D. Pugh & J.L. Sartin (2003). Leptin, tumor necrosis factor-alpha (TNF), and CD14. Journal of Animal Science, 81, 2590-2599.

Dasu, M.R., D.N. Herndon, O. Nesic & J.R. Perez-Polo (2003). IGF-I gene transfer effects on inflammatory elements present after thermal trauma. American Journal of Physiology, 285, 741-746.

Diez-Fraile A, E. Meyer & C. Burvenich (2003). Sympathoadrenal and immune system activation during the periparturient period and their association with bovine coliform mastitis. A review. Veterinary Quarterly, 25(1), 31-44.

Demas, G.E., V. Chefer, M.I. Talan & R.J. Nelson (1997). Metabolic cost of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. American Journal of Physiology, 273, R1631-1637.

Dingwell, R.T., D.F. Kelton & K.E. Leslie (2003). Management of the dry cow in control of peripartum disease and mastitis. Veterinary Clinics of North America – Food Animal Practice, 19, 235-265.

Dorshkind, K. & N.D. Horseman (2001). Anterior pituitary hormones, stress, and immune system homeostasis. Bioessays. 23(3):288-94.

Dorshkind, K. & N.D. Horseman (2000). The roles of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones in lymphocyte development and function: insights from genetic models of hormone and hormone receptor deficiency. Endocrine Reviews 21(3):292-312.

Elenkov, I.J. (2004). Glucocorticoids and the Th1/Th2 balance. Annals of the New York Academy of Science, 1024, 138-146.

Elsasser, T.H., T.J. Caperna & R. Fayer (1991). Tumor necrosis factor-alpha affects growth hormone secretion by a direct pituitary interaction. Proceedings of the Society for Experimental Biology and Medicine. 198(1):547-54.

Elsasser, T.H., S. Kahl, C. MacLeod, B. Nicholson, J.L. Sartin & C. Li (2004) Mechanisms underlying growth hormone effects in augmenting nitric oxide production and protein tyrosine nitration during endotoxin challenge. Endocrinology, 145, 3413-3423.

Elsasser, T.H. (2003). Insulin-like growth factor-1: a traffic control devise on the road to tissue recovery. American Journal of Physiology, 285, R723-724.

Elsasser, T.H., R. Fayer, T.S. Rumsey & G. Hartnell (1994). Recombinant bovine somatotropin blunts plasma tumor necrosis factor-α, cortisol, and thromboxane-B_2 responses to endotoxin *in vivo*. Endocrinology, 134, 1082-1088.

Elsasser, T.H. & S. Kahl (2002). Adrenomedullin has multiple roles in disease stress: Development and remission of the inflammatory response. Microscopy Research and Technique, 57, 120-129.

Elsasser, T.H., S. Kahl, A. Martínez, L.M. Montuengs, R. Pio & F. Cuttitta. (1999). Adrenomedullin binding protein in the plasma of multiple species: Characterization by radioligand blotting. Endocrinology, 140, 4908-4911.

Elsasser, T.H., S. Kahl, T.S. Rumsey & J.W. Blum (2000). Modulation of growth performance in disease: reactive nitrogen compounds and their impact on cell proteins. Domestic Animal Endocrinology, 19, 75-84.

Elsasser, T.H., S. Kahl, J.L. Sartin, R. Fayer, A. Martínez & F. Cuttitta (2003). Muscle protein nitration patterns during chronic subclinical intramuscular parasitism: Colocalization to fiber-type and ubiquitin. Journal of Animal Science 81 (Suppl. 1): 84.

Elsasser, T.H., S. Kahl, N.C. Steele & T.S. Rumsey (1997). Nutritional modulation of the somatotropic axis – cytokine relationships in cattle: A brief review. Comparative Biochemistry and Physiology, 116, 209-221.

Elsasser, T.H., K. Klasing, N. Filipov & F. Thompson (2000). The metabolic consequences of stress: targets for stress and priorities of nutrient use. In: G. Moberg & J. Mench, (eds.) The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI Publishing, New York, pp. 77-110.

Elsasser, T.H., R.W. Rosebrough, T.S. Rumsey & W.M. Moseley (1996). Hormonal and nutritional modulation of arginase activity in growing cattle. Domestic Animal Endocrinology, 13, 219-228.

Elsasser, T.H., T.S. Rumsey & A.C. Hammond (1989). Influence of diet on basal and growth hormone-stimulated plasma concentrations of IGF-I in beef cattle. Journal of Animal Science 67(1):128-41.

Elsasser, T.H., J.L. Sartin, A. Martínez, S. Kahl, L. Montuenga, R. Pio, R. Fayer, M. Miller & F. Cuttitta (1999). Underlying disease stress augments plasma and tissue adrenomedullin (AM) responses to endotoxin: Colocalized increases in AM and inducible nitric oxide synthase within pancreatic islets. Endocrinology, 140, 5402-5410.

Enevoldsen, C. & J.T. Sorensen (1992). Effects of dry period length on clinical mastitis and other major clinical health disorders. Journal of Dairy Science, 75(4), 1007-1014.

Evain-Brion, D. & A. Malassine (2003). Human placenta as an endocrine organ. Growth hormone and IGF Research, 13 (suppl a) S34-37.

Fujimoto T., R.C. Savani, M. Watari, A.J. Day & J.F. Strauss 3rd (2002). Induction of the hyaluronic acid-binding protein, tumor necrosis factor-stimulated gene-6, in cervical smooth muscle cells by tumor necrosis factor-alpha and prostaglandin E(2). American Journal of Pathology, 160(4), 1495-1502.

Goff, J.P. & R.L. Horst (1997). Physiological changes at parturition and their relationship to metabolic disorders. Journal of Dairy Science, 80, 1260-1268.

Gluckman, P.D., S.V. Sizonenko & N.S. Bassett (1999). The transition from Fetus to neonate – an endocrine perspective. Acta Pediatrics Scandinavia, 88, 7-11.

Gorczynski, R.M., S. Hadidi, G. Yu & D.A. Clark (2002). The same immunoregulatory molecules contribute to successful pregnancy and transplantation. American Journal of Reproductive Immunology, 48, 18-26.

Gow, A.J., C.R. Farkouh, D.A. Munson, M.A. Posencheg & H. Ischiropoulos (2004). Biological significance of nitric oxide-mediated protein modifications. American Journal of Physiology, 287(2), L262-268.

Hammond, J. (1952). Physiological limits to intensive production in animals. British Agriculture Bulletin, 4, 222-225.

Hansen, T.K. (2003). Growth hormone and mannan binding lectin: emerging evidence for hormonal regulation of innate immunity. Minerva Endocrinologica, 28, 75-84.

Heemskerk, V.H., M.A. Daemen & W.A. Buurman (1999). Insulin-like growth factor -1 (IGF-1) and growth hormone (GH) in immunity and inflammation. Cytokine Growth Factor Reviews, 10, 5-14.

Hernandez, A., N. Karrow & B.A. Mallard (2003). Evaluation of immune responses of cattle as a means to identify high or low responders and use of a human microarray to differentiate gene expression. Genetics Selection and Evolution, 35 Suppl 1, S67-81.

Hesse, M., M. Modolell, A.C. La Flamme, M. Schito, J.M. Fuentes, A.W. Cheever, E.J. Pearce & T.A. Wynn (2001). Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. Journal of Immunology, 167, 6533-6544.

Ingvartsen, K.L. & J.B. Andersen (2000). Integration of metabolism and intake regulation: a review focusing on periparturient animals. Journal of Dairy Science, 83, 1573-1597.

Ingvartsen, K.L & Y.R. Boisclair (2001). Leptin and the regulation of food intake, energy homeostasis and immunity with special focus on periruminant ruminants. Domestic Animal Endocrinology, 21, 215-250.

Ingvartsen, K.L., R.J. Dewhurst & N.C. Friggens (2003). On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production disease in cattle? A position paper. Livestock Production Science, 83, 277-308.

Ischiropoulos H. (2003). Biological selectivity and functional aspects of protein tyrosine nitration. Biochemical and Biophysical Research Communication, 305, 776-783.

Jacob, S.K., V. Ramnath, P.T. Philomina, K.V. Raghunandhanan & A. Kannan (2001). Assessment of physiological stress in periparturient cows and neonatal calves. Indian J Physiol Pharmacol. 45(2):233-8.

Jahnke, G.D., M.J. Miller, A. Martínez, L. Montuenga & F. Cuttitta (1997). Adrenomedullin expression in the mouse mammary gland: evidence for the mature form in milk. Journal of Molecular Endocrinology, 19, 279-289.

Johnson, R.W., S. Arkins, R. Dantzer & K.W. Kelley (1997). Hormones, lymphohemopoietic cytokines and the neuroimmune axis. Comperative Biochemistry and Physiology, 116, 183-201.

Kahl, S, T.H. Elsasser & J.W. Blum (1997). Nutritional regulation of plasma tumor necrosis factor-alpha and plasma and urinary nitrite/nitrate responses to ebdotoxin in cattle. Proceedings of the Society for Experimental Biology and Medicine, 215, 370-376.

Kahl, S. & T.H. Elsasser (2004). Endotoxin challenge increases xanthine oxidase activity in cattle: Effect of growth hormone and vitaqmin E treatment. Domestic Animal Endocrinology, 26, 315-328.

Kawanaka, H., M.K. Jones, I.L. Szabo, D. Baatar, R. Pai, K. Tsugawa, K. Sugimachi, I.J. Sarfeh & A.S. Tarnawski (2002). Activation of eNOS in rat portal hypertensive gastric mucosa is mediated by TNF-alpha via the PI 3-kinase-Akt signaling pathway. Hepatology, 35, 393-402.

Kehrli, M.E., J.L. Burton, B.J. Nonnecke & E.K. Lee (1999). Effects of stress on leukocyte trafficking and immune responses: implications for vaccination. Advances in Veterinary Medicine, 41, 61-81.

M.E. Kehrli, J.D. Neill, C. Burvenich, J.P. Goff, J.D. Lippolis & T.A. Reinhardt (2004). Energy and protein effects on the immune system. In: K.ejrsen, T. Hvelplund and M.O. Nielsen (eds.), Ruminant physiology: Digestion, metabolism and impact of nutrition on gene expression, immunology and stress, Wageningen Academic Publishers, The Netherlands. pp. 455-471.

Kelley, K.W. (1990). The role of growth hormone in the modulation of the immune response. Annals New York Academy of Science, 594, 95-103.

Kitamura, K., K. Kangawa, M. Kawamoto, Y. Ichiki, S. Nakamura, H. Matsuo & T. Eto (1993). Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochemical Biophysical Research Communication, 30;192, 553-560.

Klasing, K.C. & C.C. Calvert (2000). The care and feeding of an immune system: an analysis of lysine needs. In: G.E. Lobley, A. White & J.C. MacRae (eds.) Proceedings of the VIIIth International Symposium on Protein Metabolism and Nutrition, pp. 253-264. Wageningen Press, Wageningen.

Koch, C.A. & J.L. Platt (2003). Natural mechanisms for evading graft rejection: The fetus as an allograft. Speinger Seminars in Immunopathology, 25, 95-117.

Koets, A.P., N. De Schwartz, P. Tooten, M. Kankofer, J.M. Broekhuijsen-Davies, V.P. Rutten, L.A. Van Leengoed, M.A. Taverne & E. Gruys (1998). Release of proinflammatory cytokines related to luteolysis and the periparturient acute phase response in prostaglandin-induced parturition in cows. Theriogenology. 49(4):797-812.

Komine, K., T. Kuroishi, Y. Komine., K. Watanabe, J. Kobayashi, T. Yamaguchi, S. Kamata & K. Kamagai (2004). Induction of nitric oxide production mediated by tumor necrosis factor-α on Stasphylococcal Enterotoxin C-stimulated bovine mammary gland cells. Clinical and Diagnostic Laboratory Immunology, 11, 203-210.

Lacasse, P., V.C. Far, S.R. Davis & C.G. Prosser (1996). Local secretion of nitric oxide and the control of mammary blood flow. Journal of Dairy Science, 79(8), 1369-74.

Macri, C.J., A. Martínez, T.W. Moody, K.D. Gray, M.J. Miller, M. Gallagher & F. Cuttitta (1996). Detection of adrenomedullin , a hypotensive peptide , in amniotic fluid and fetal membranes. American Journal of Obstetrics and Gynacology, 175, 906-911.

Madsen, S.A., P.S. Weber & J.L. Burton (2002). Altered expression of cellular genes in neutrophils of periparturient dairy cows. Vet Immunol Immunopathol. 86(3-4):159-75.

Mallard, B.A., J.C. Dekkers, M.J. Ireland, K.E. Leslie, S. Sharif, C.L. Vankampen, L. Wagter & B.N. Wilkie (1998). Alterations in immune responsiveness during the peripartum period and its ramifications on dairy cow and calf health. Journal of Dairy Science, 81, 585-595.

Mallard, B.A., L.C. Wagter, M.J. Ireland & J.C. Dekkers (1997). Effects of growth hormone, insulin-like growth factor-I, and cortisol on periparturient antibody response profiles of dairy cattle. Veterinary Immunology and Immunopathology, 60, 61-76.

Martínez, A., E.J. Unsworth & F. Cuttitta (1996). Adrenomedullin-like immunoreactivity in the nervous system of the starfish Marthasterias glacialis. Cell Tissue Research 283(2):169-72.

Martínez, A., M.J. Miller, L. Montuenga & F. Cuttitta (1998a). Adrenomedullin regulates Hormone secretion. In: A. Martínez & F. Cuttitta (eds.) Adrenomedullin. IOS Press, Washington, D.C., pp. 185-196.

Martínez, V. H. Kaneko & Y. Taché (1998b). Adrenomedullin and regulation of gastric function. In: A. Martínez & F. Cuttitta (eds.) Adrenomedullin. IOS Press, Washington, D.C., pp. 249-266.

Martínez, A., R. Pio, P.F. Zipfel & F. Cuttitta (2003). Mapping of the adrenomedullin binding domains in human complement factor H. Hypertension Research, 26(suppl) ; S55-59.

Mattacks, C.A., D. Sadler & C.M. Pond (2003). The cellular structure and lipid/protein composition of adipose tissue surrounding chronically stimulated lymph nodes in rats. Journal of Anatomy, 202, 552-561.

Meglia G.E., A. Johannisson, L. Petersson & K.P. Waller (2001). Changes in some blood micronutrients, leukocytes and neutrophil expression of adhesion molecules in periparturient dairy cows. Acta Veterinaria Scandinavia, 42(1), 139-150.

Miyashita, K., H. Itoh, N. Sawada, Y. Fukunaga, M. Sone, K. Yamahara, T. Yurugi-Kobayashi, K. Park & K. Nakao (2003). Adrenomedullin provokes endothelial Akt activation and promotes vascular regeneration both *in vitro* and *in vivo*. FEBS Letters 544, 86-92.

McGuire, M.A., D.A. Dwyer, D.E. Bauman & D.F. Smith (1998). Insulin-like growth factors in plasma and afferent mammary lymph of lactating cows deprived of feed or treated with bovine somatotropin. Journal of Dairy Science, 81(4), 950-957.

Minamino, N., K. Kangawa & H. Matsuo (2000). Adrenomedullin: a new peptidergic regulator of the vascular function. Clinical Hemorheolgy and Microcirculation, 23, 95-102.

Naranjo, W.M., S. Yakar, M. Sanchez-Gomez, A.U. Perez, J. Setser & D. LeRoith (2002). Protein calorie restriction affects nonhepatic IGF-I production and the lymphoid system: studies using the liver-specific IGF-I gene-deleted mouse model. Endocrinology, 143(6), 2233-2241.

Oetzel, G.R. (2000). Management of dry cows for the prevention of milk fever and other mineral disorders. Vet Clin North Am Food Anim Pract. 16(2):369-86, vii.

Pfaffl, M.W, S.L. Wittmann, H.H.D. Meyer & R. Bruckmaier (2003). Gene expression of immunologically important factors in blood cells, milk cells and mammary tissues of cows. Journal of Dairy Science, 86, 538-545.

Pfaffl, M., F. Schwarz & H. Sauerwein (1998). Quantification of insulin-like growth factor-1 (IGF-1) mRNA: modulation of growth intensity by feeding results in inter- and intra-tissue-specific differences of IGF-1 mRNA expression in steers. Experimental and Clinical Endocrinology and Diabetes, 106(6), 514-21

Pio, R., A. Martínez, T. Elsasser & F. Cuttitta (2000). Presence of immunoreactive adrenomedullin in human and bovine milk. Peptides, 21:1859-1863.

Pio, R., A. Martínez, E.J. Unsworth, J.A. Kowalak, J.A. Bengoechea, P.F. Zipfel, T.H. Elsasser & F. Cuttitta (2001). Complement factor H is a serum-binding protein for adrenomedullin, and the resulting complex modulates the bioactivities of both partners. Journal of Biological Chemistry, 276, L12292-L122300.

Pond, C.M. (2003). Paracrine interactions of mammalian adipose tissue. Journal of Experimental Zoology, Part "A", Comparative Experimental Biology, 295, 99-110.

Pond, C.M. & C.A. Mattacks (2001). The activation of the adipose tissue associated with lymph nodes during the early stages of the immune response. Cytokine, 17, 131-139.

Rogers, A.M., I. Boime, J. Connolly, J.R. Cook & J.H. Russell (1998). Maternal-fetal tolerance is maintained despite transgene-driven trophoblast expression of MHC class I, and defects in Fas and its ligand. European Journal of Physiology, 28, 3479-3487.

Rumsey, S. & A.C. Hammond (1990). Responses to an estrogenic growth promoter in beef steers fed varying nutritional regimens. Journal of Animal Science, 77, 2865-2872.

Saito, Y., C. Nakagawa, H. Uchida, F. Sasaki & H. Sakakibara (2001). Adrenomedullin suppresses fMLP-induced upregulation of CD11b of human neutrophils. Inflammation, 25, 197-201.

Salem, M.L. (2004). Estrogen, a double-edged sword: modulation of TH1- and TH2-mediated inflammations by differential regulation of TH1/TH2 cytokine production. Curr Drug Targets Inflamm Allergy. 3(1):97-104.

Sartin, J.L., T.H. Elsasser, S. Kahl, J. Baker, J.A. Daniel, D.D. Schwartz, B. Steele & B.K. Whitlock (2003). Estradiol plus progesterone treatment modulates select elements of the proinflammatory cytokine cascade in steers: attenuated nitric oxide and thromboxane B2 production in endotoxemia. Journal of Animal Science, 81(6), 1546-1551.

Schmitz, S., M.W. Phaffl, H.H.D. Meyer & R. Bruckmaier (2004). Short-term changes of mRNA expressions of various inflammatory factors and milk proteins in mammary tissue during LPS-induced mastitis. Domestic Animal Endocrinology, 26, 111-126.

Schwartz, J. & I.C. McMillen (2001). Fetal-hypothalamic-pituitary-adrenal axis on the road to parturition. Clinical and Experimental Pharmacology and Physiology, 28, 108-112.

Shimosawa, T., H. Matsui, G. Xing, K. Itakura, K. Ando & T. Fujita (2003). Organ-protective effects of adrenomedullin. Hypertension Research, 26, (suppl) S109-112.

Sites, D.P. & P.K. Siiteri (1983). Steroids as immunosuppressants in pregnancy. Immunology Reviews, 75, 117-138.

Smith, U., M. Axelsen, E. Carvalho, B. Eliasson, P.A. Jansson & C. Wesslau (1999). Insulin signaling and action in fat cells: associations with insulin resistance and type 2 diabetes. Annals of the New York Academy of Science, 892, 119-126.

Soeters, P.B., M.M. Hallemeesch, M.J. Bruins, H.M. van Eijk & N.E. Deutz (2002). Quantitative in vivo assessment of arginine utilization and nitric oxide production in endotoxemia. American Journal of Surgery, 183(4), 480-488.

Sorensen, J.T & C. Enevoldsen (1991). Effect of dry period length on milk production in subsequent lactation. Journal of Dairy Science, 74(4), 1277-1283.

Sternberg, E.M. (2001). Neuroendocrine regulation of autoimmune/inflammatory disease. J Endocrinol. 169(3):429-35.

Taylor, M.M. & W.K. Samson (2002). Adrenomedullin and the integrative physiology of fluid and electrolyte balance. Microscopy Research and Technique. 57(2):105-9.

van Buul-Offers, S.C. & R. Kooijman (1998). The role of growth hormone and insulin-like growth factors in the immune system. Cellular and Molecular Life Science, 54, 1083-1094.

van Hooijdonk, A.C., K.D. Kussendrager & J.M. Steijns (2000). *In vivo* antimicrobial and antiviral activity of components in bovine milk and colostrums involved in non-specific defense. British Journal of Nutrition, 84, S127-134.

Wei, C.L., W.M. Hon, K.H. Lee, M. Mori, T. Gotoh & H.E. Khoo (2002). Induction of arginase II in livers of bile duct-ligated rats. Biochem Pharmacol., 63(6), 1043-1050.

Welsch, U., P. Unterberger, E. Hofter, F. Cuttitta & A. Martínez (2002). Adrenomedullin inmammalian and human skin glands including mammary gland. Acta Histochemica, 104, 65-72.

Wren, G. (2003). Immunology of the calving cow: Part II. Dairy Herd Management. http://www.dairyherd.com/news_editorial.asp?pgID=675&ed_id=3097.

Yin, H., L. Chao & J. Chao (2004). Adrenomedullin protects against myocardial apoptosis afterischemia/reperfusion through activastion of Akt-GSK signaling. Hypertension, 43, 109-116.

Zaks-Zilberman, M., C.A. Salkowski, T. Elsasser, F. Cuttitta & S.N. Vogel (1998) Induction of adrenomedullin mRNA and protein by lipopolysaccharide and paclitaxel (Taxol) in murine macrophages. Infection and Immunity. 66(10):4669-75.

Zudaire, E., F. Cuttitta & A. Martínez (2003). Regulation of pancreatic physiology by adrenomedullin and its binding protein. Regulatory Peptides, 112, 121-130.

Energy and protein effects on the immune system

M.E. Kehrli, Jr.[1], J.D. Neill[1], C. Burvenich[2], J.P. Goff[3], J.D. Lippolis[3], T.A. Reinhardt[3] and B.J. Nonnecke[3]
[1]National Animal Disease Center-USDA-ARS, Virus and Prion Diseases of Livestock Research Unit, 2300 Dayton Avenue, Ames, Iowa, 50010 USA
[2]Universiteit Gent, Vakgroep Fysiologie, Biochemie en Biometrie, Salisburylaan 133, 9820 Merelbeke, Belgium
[3]National Animal Disease Center-USDA-ARS, Periparturient Diseases of Cattle Research Unit, 2300 Dayton Avenue, Ames, Iowa, 50010 USA

Abstract

We will provide an overview of the effects of energy and protein status on the immune system, with a particular focus on the periparturient dairy cow. Recent studies have shown a significant component of the leukocyte proteome is committed to energy metabolism and cell signalling machinery. The various proteins involved in enabling and maintaining leukocyte function represent a demand on the host's protein metabolism. As part of this discussion we will focus on metabolic challenges facing the transition cow and how milk production influences metabolism and immune function. We also know there is considerable genetic control over the immune system capacity to function. There is as much genetic difference in immune function during the periparturient period between cows of average milk production capability as there is between cows of high milk production capacity. Therefore, very high milk production will not likely dictate that a cow will experience a greater magnitude or duration of immune suppression than a lower producing cow. It is more likely the inability of individual cows to adapt to the metabolic demands of milk production dictates the degree and duration of immune suppression experienced at calving.

Keywords: immunity, protein, energy, metabolism

Introduction

Generally, the dry period of the cow is considered as a period of rest between two lactations. However, all is not restful during the dry period as considerable changes in fetal growth, mammary tissue remodeling and nutritional demands occur. Today, transition cows are typically referred to as cows in the second half of the dry period (2 to 4 weeks prepartum) through 2 to 4 weeks postpartum. This transition period represents a particularly challenging period in the production cycle of a modern dairy cow. In terms of physiological regulation, this transition period is very critical because food intake is reduced around parturition whilst nutrient demands for support of growth of the calf, and initiation of milk protein, fat and lactose synthesis are increased. Moreover, the diet of most dairy cows changes abruptly at calving from being predominantly forage to a diet containing high levels of concentrates and high quality forage. Inadequate physiological adaptation and/or nutrition during the transition from a pregnant, non-lactating status to a non-pregnant, lactating condition may result in a disastrous experience for the cow. Most metabolic diseases of dairy cows - milk fever, ketosis, retained placenta, and displacement of

the abomasum - occur within the first 2 weeks of lactation. In addition to metabolic diseases, the majority of infectious disease experienced by the dairy cow becomes clinically apparent during the first 2 wk of lactation. This is especially true for mastitis, but also includes diseases such as Johne's disease and salmonellosis.

Mastitis begins with the establishment of an intramammary infection (IMI). Many bacteria that cause bovine mastitis, such as coliforms, are opportunistic pathogens. Opportunistic bacterial infections occur when the integrity of native host defenses is breached and often are indicative of predisposing immunosuppression in the host animal. The prepartum mammary gland is highly susceptible to new infections (Smith *et al*., 1985b). Moreover, a high percentage of intramammary infections present at parturition develop into clinical mastitis during the first 60-70 days of lactation (Malinowski *et al*., 1983, Smith *et al*., 1985b, Smith *et al*., 1985a, Hogan *et al*., 1989). Many years ago, we Kehrli *et al.* (Kehrli and Goff, 1989, Kehrli *et al*., 1989a, Kehrli *et al*., 1989b), hypothesized that immunosuppression occurs during the periparturient period and therefore, predisposes the dairy cow to new infections and may result in a heightened susceptibility to IMI and/or the progression of subclinical mastitis into clinical disease.

Immune function status of periparturient dairy cattle

For nearly 30 years it has been recognized the periparturient bovine immune system is less capable of battling pathogens and exhibits a wide range of immunological dysfunction (Guidry *et al*., 1976, Newbould, 1976, Wells *et al*., 1977), manifest as impaired neutrophil and lymphcyte capabilities. Circulating lymphocytes have reduced capacity to produce interferon-γ (IFN-γ (Ishikawa *et al*., 1994) and there are reduced IFN-γ and interleukin-2 levels in lacteal secretions of periparturient cows (Sordillo *et al*., 1991). This immunosuppression is most evident in the Th1 branch of lymphocyte activity and may be essential in preventing unwanted immune reactions against self and paternal antigens exposed to the mother's immune system as a result of tissue damage in the reproductive tract during parturition. Lymphocytes exposed to 1α, 25-dihydroxyvitamin D_3 produce less IFN-γ and IL-2, and more IL-4, IL-5, and IL-10 (Daynes *et al*., 1996). The parturient surge in 1α, 25-dihydroxyvitamin D_3 may therefore contribute to the reduced capacity of bovine lymphocytes to produce IFN-γ (Sordillo *et al*., 1991, Ametaj *et al*., 1996, Ametaj *et al*., 2000). Leukocyte trafficking patterns also change in periparturient cows; as the percentage of T cells declines from ~45% of circulating lymphocytes in mid-lactation cows to ~20% in periparturient cows (Shafer-Weaver *et al*., 1996). The proportion of $CD4^+$ cells in blood and mammary parenchyma also declines postpartum, which is consistent with decreased IFN-γ secretion by lymphocytes and decreased IFN-γ in lacteal secretions of periparturient cows (Sordillo *et al*., 1991, Ishikawa *et al*., 1994, Shafer-Weaver *et al*., 1996, Shafer-Weaver and Sordillo, 1997, Yang *et al*., 1997). The functional role of $CD8^+$ lymphocytes during the postpartum period is most likely of a suppressor nature because IL-4 mRNA is the main cytokine detectable in mononuclear cells from postpartum dairy cows while IFN-γ is the main cytokine detected from cows in middle to later stages of lactation (Shafer-Weaver and Sordillo, 1997).

The worldwide consistency of findings on periparturient immunosuppression has been remarkable. For example, production of reactive oxygen species (ROS) of neutrophils isolated from blood were evaluated in two longitudinal studies in dairy cows from 3 weeks before until

5 weeks after calving, in the United States and in Europe. In both studies, a significant decrease in oxidative burst activity of neutrophils was observed (Dosogne *et al*., 1999). Interestingly, in both the U.S. studies and European studies, ingestion of bacteria and ROS production were inversely correlated. While the phagocytic ingestion capacity increased during the two weeks before parturition, ROS levels were decreasing. Immature neutrophils cannot be held responsible for this inverse correlation, since they are not present in circulation prior to calving. Therefore, it might be hypothesized that the reduced ROS production of blood neutrophils around parturition underlies the selective depression of activation pathways involved in oxidative burst generation without affecting the ingestion capacity. Furthermore, the increased capacity for ingestion of pathogens by neutrophils may simply be a function of the fact that the cellular energy required to support the respiratory burst of oxidative metabolism is not used due to defects in that pathway, thus allowing neutrophils to deplete their energy stores by ingesting excessive numbers of bacteria. The drop in neutrophil ROS production around parturition is more pronounced in multiparous cows (Mehrzad *et al*., 2002). The pronounced reduction in neutrophil oxidative burst capacity and viability in milk neutrophils of multiparous cows may be involved in the underlying mechanisms that make these animals more susceptible to periparturient infectious diseases.

A cause and effect relationship between a faltering innate and adaptive immune system and the development of IMI may be difficult to prove. However, the severity of *E. coli* mastitis is known to be correlated with the decrease in circulating neutrophil ROS production before IMI (Heyneman *et al*., 1990). Recently, Mehrzad *et al.* (unpublished data, 2004) found that in experimentally challenged cows with intramammary *E. coli*, there was an inverse relationship between pre-infection milk neutrophil ROS production and intramammary bacterial growth. In mild and moderately affected cows, the pre-infection blood and milk neutrophil ROS production was ~2-fold higher than that of severely affected cows. The probability of severe disease increased with decreasing pre-infection neutrophil function. Today it is widely accepted that severity of *E. coli* mastitis is mainly determined by cow factors. In this way physiology comes very close to pathology (Burvenich *et al*., 2003, Burvenich *et al*., 2004).

There is a remarkable variability in the clinical expression and complications of coliform mastitis around parturition, ranging from clinical severe to moderate and mild (Hill *et al*., 1979, Hill, 1981, Heyneman *et al*., 1990, Vandeputte-Van Messom *et al*., 1993, Burvenich *et al*., 1994, Shuster *et al*., 1996, Hirvonen *et al*., 1999, Burvenich *et al*., 2003). This is in sharp contrast with the more moderate clinical expression of coliform mastitis during established lactation (Burvenich *et al*., 1994, Shuster *et al*., 1996). There is also a tendency to see more severe clinical coliform mastitis cases in multiparous cows (Van Werven *et al*., 1997, Mehrzad *et al*., 2001, Mehrzad *et al*., 2002, Burvenich *et al*., 2003, Vangroenweghe *et al*., 2004). Delays in the inflammatory response in cows with peracute coliform mastitis have been reported in certain cows shortly after calving (Hill *et al*., 1979, Hill, 1981, Heyneman *et al*., 1990, Vandeputte-Van Messom *et al*., 1993).

Bovine leukocyte adhesion molecules such as L-selectin (CD62L) and the β_2-integrins play a key role in the initial contact and in the subsequent strong adhesion to post-capillary venules, respectively. Activated neutrophil egress, is subsequently mediated by chemotactic gradients generated by the local tissue response to infection (Burvenich *et al*., 1994). Studies of periparturient cows during the first few hours after calving have revealed a transient loss of expression of critical

neutrophil adhesion molecules (CD62L) (Lee and Kehrli, 1998, Kimura *et al*., 1999, Monfardini *et al*., 2002). The loss of CD62L on neutrophils after parturition is associated with elevated cortisol (Weber *et al*., 2001, Weber *et al*., 2004). Glucocorticoid administration to cattle is well documented to cause shedding of CD62L from neutrophils (Burton and Kehrli, 1995, Burton *et al*., 1995). The net effect of reduced neutrophil CD62L expression would be a transient loss in the efficiency of neutrophil immune surveillance and egress into infected tissues. Should a cow experience a large demand for neutrophil egress into the uterus or mammary gland when immune surveillance is transiently impaired, a severe disease may occur as a result of a delayed inflammatory response. A delay in neutrophil arrival in lacteal secretions of just 20 minutes is not an insignificant effect, considering that *E. coli* can double its numbers every 20 minutes. A 1-hour delay in neutrophil recruitment into the mammary gland could result in an 8-fold larger number of *E. coli* to kill and that much more endotoxin to detoxify. This delay in neutrophil recruitment has been reported in many studies (Hill *et al*., 1979, Hill, 1981, Heyneman *et al*., 1990, Vandeputte-Van Messom *et al*., 1993). We have also observed an occasional cow that appears to not recognize the presence of *E. coli* in the mammary gland and hence allows the organism to grow to large numbers that ultimately result in a fatal case of mastitis (Shuster *et al*., 1996). Consistent with these clinical findings, postpartum cows in our studies were much more susceptible to bacterial growth in milk (100-times greater numbers of *E. coli* were found in postpartum versus midlactation cows) (Shuster *et al*., 1996). However, as a general rule, we found most 7 to 10-day postpartum cows were capable of recruiting neutrophils into the mammary gland as fast as midlactation cows (Shuster *et al*., 1996). Impairment of other neutrophil functions (e.g., oxidative metabolism and myeloperoxidase activity) in postpartum cows is a likely contributor to this increased bacterial growth (Kehrli and Goff, 1989, Kehrli *et al*., 1989b, Cai *et al*., 1994, Detilleux *et al*., 1995). Postpartum cows also experienced a more severe episode of coliform mastitis that is likely due to the much greater endotoxin burden associated with the greater bacterial numbers (Shuster *et al*., 1996). Another factor contributing to severity of coliform mastitis in postpartum cows is a reduction of acyloxyacyl hydrolase activity in circulating neutrophils (Dosogne *et al*., 1998). This enzyme is critical in detoxification of endotoxin (McDermott *et al*., 1991a, McDermott *et al*., 1991b). In this context it is important to mention again the variability of the inflammatory response during coliform mastitis around parturition, ranges from severe to moderate to mild, and that this variability is unpredictable and differs for reasons that remain unravelled (Burvenich *et al*., 2003).

The metabolic demands of lactation impact the ability of the postpartum cow to manage her metabolism, as well as her ability to recover from periparturient immunosuppression. This has been shown in studies utilizing mastectomized cows (Kimura *et al*., 1999, Goff *et al*., 2002, Kimura *et al*., 2002, Nonnecke *et al*., 2003, Stabel *et al*., 2003), as well as with studies in periparturient cows with rumen cannulas where additional total mixed rations were manually stuffed into their rumens with refused feed to maintain dry matter intake of 2% body weight per day before calving and 2.5% body weight per day after calving (Stabel *et al*., 2003). However, it is equally important to note that genetics has significant effects on the metabolic and immunologic capabilities of dairy cattle. Dairy cattle selected for high milk production have considerable genetic variance in their immunological capabilities (Detilleux *et al*., 1994). This genetic variance is greater than the difference in immune competence between cows selected for high and average levels of milk production, therefore, some high producing dairy cattle can have high values for immune

competence during this stressful period. This is encouraging in that selection for higher milk production is possible without detriment to immune function. The findings that high milk production cows had significantly higher number of neutrophils and higher neutrophil ability to perform the metabolic burst is important because neutrophils provide front line defenses that can be rapidly mobilized and activated against infectious or toxic agents (van der Valk and Herman, 1987). In a recent study, milk production level did not affect host resistance in dairy cows, as measured by the severity of experimental *E.coli* mastitis. Even in a situation where cows were metabolically stressed by overfeeding, high producers were as able as low producers to cope with the demands of milk production, without consequences for host resistance (Kornalijnslijper *et al*., 2003). In dairy cattle, significant genetic influence has been found for several immunologic traits: neutrophil phagocytosis and lymphocyte responses to mitogens (Kehrli *et al*., 1991, Weigel *et al*., 1991), serum Ig and lysozyme levels (Lie *et al*., 1986), and serum hemolytic complement levels (Lie *et al*., 1983).

Energy and protein status of periparturient dairy cattle

It is well recognized that dairy cattle experience a negative energy and protein balance as a result of the sudden onset of lactation and insufficient dietary intake of nutrients. This causes an array of metabolic adjustments to be made by the cow, placing considerable demands on liver function. Fatty liver is a metabolic disease of high producing dairy cows that is believed to negatively impact milk production, disease resistance, and reproductive performance (Bobe *et al*., 2004). Although a debate exists over which occurs first, similar to the chicken and the egg, most scientists believe the existence of a fatty liver is a prelude to ketosis. Several theories exist to describe the cause of fatty liver development in the bovine.

Over the past 10 years, most research on ruminant tissue lipid metabolism has focused on fatty acid peroxisomal oxidation in the liver and in muscles, regulation of lipid metabolism by leptin and the effects of activation of selected nuclear receptors by long-chain fatty acids or by phytol metabolites derived from chlorophyll (Hocquette and Bauchart, 1999). High levels of non-esterified fatty acid (NEFA) uptake by the liver in high-yielding dairy cows in early lactation leads to triglyceride infiltration of the hepatocytes (fatty liver). Some research indicates this is due to the low capacity of the ruminant liver to synthesize and secrete VLDL particles in comparison with primates or rodents. This abnormality in hepatic fatty acid metabolism involves defects in apolipoprotein B synthesis and low availability of apolipoproteins and lipids for VLDL packaging (Gruffat-Mouty *et al*., 1999). A low rate of synthesis of apolipoprotein B does not appear to limit VLDL secretion but rather a defect in VLDL assembly and/or secretion appears to be the rate limiting step in VLDL secretion in the ruminant liver. Methods to enhance secretion of VLDL by liver cells may aid in the prevention of fatty liver as a prelude to clinical ketosis.

Ketosis is a metabolic disorder in early lactation dairy cows characterized by hypoglycemia and hyperketonemia. It is caused by insufficient blood glucose to support metabolic needs associated with the onset of heavy lactation after calving and when feed intake is not maximal. Lack of blood glucose induces a decline in plasma insulin, an increase in fat mobilization from adipose tissue and an increase in hepatic ketogenesis. Increased fatty acid mobilization leads to fatty infiltration of the liver and this accumulation of fat in the liver precede the onset of clinical ketosis. Fatty

infiltration of the liver occurs when fatty acid uptake and triacylglycerol (TAG) synthesis exceed the capacity of the liver to oxidize fatty acids or to hydrolyze and export TAG as VLDLs. The bovine liver at this time has a relatively limited capacity to synthesize and export VLDLs. It is believed that prevention of fatty liver development would favorably impact the incidence of clinical ketosis. Glucagon treatment rapidly mobilizes liver glycogen and decreases hepatic TAG, blood nonesterified fatty acid and β-hydroxybutyrate levels (Hippen *et al*., 1999a, Hippen *et al*., 1999b, She *et al*., 1999, Hippen, 2000).

The cow may also accumulate fat in the liver due to failure to transport fats from the liver. Excessive triglyceride accumulation in the liver and elevated NEFA levels in disorders such as ketosis, displaced abomasum and fatty liver, coincide with low apolipoprotein B_{100} ($ApoB_{100}$) levels [see review by (Katoh, 2002)] and low $ApoB_{100}$ mRNA expression in liver (Bernabucci *et al*., 2004). Apolipoprotein B_{100} is critical for VLDL secretion in transition cows and many studies have reported low serum $ApoB_{100}$ levels in postpartum cows. Many theories exist around the limiting factors that lead to ketosis and fatty liver. Mazur *et al.* proposed that impaired synthesis of $ApoB_{100}$ may decrease its availability for lipoprotein synthesis and consequently result in excessive triglyceride accumulation in hepatocytes (Mazur *et al*., 1992). Therefore, a key to preventing fatty liver may be to understand what limits hepatic VLDL secretion in the periparturient cow and take steps to increase possibly by increasing $ApoB_{100}$ levels. A possible key to preventing fatty liver is to understand what limits hepatic VLDL secretion in the periparturient cow and take steps to increase it.

Drackley *et al*., (Drackley, 1999, Drackley *et al*., 2001) suggest it is necessary for the transition dairy cow to induce hepatic mitochondrial β-oxidation of fatty acids via the peroxisome proliferator-activated receptors (PPARs). Mitochondrial β-oxidation in the liver provides energy to cells and can provide ATP needed for gluconeogenesis. Once the oxidative capacity of the liver is exceeded the fatty acids are converted to ketones in order to prevent a toxic accumulation of acetyl-CoA. The substrate supply for mitochondrial β-oxidation includes NEFAs mobilized from adipose tissue. NEFAs rise dramatically after calving to meet the energy demands of lactation. It appears there is natural induction (via PPARs) of enzymes of fatty acid metabolism that adapt oxidative metabolism of the cow to use fatty acids ~2 weeks after calving. Drackley has shown dietary manipulation in prepartum cows can initiate this induction sooner than what occurs naturally; however, dietary management is not always practical nor easy to implement for transition cows. Many other theories exist around how to better manage the metabolic changes in the transition cow. Central to all these theories is to maximize feed intake and minimize serum NEFA levels around calving to maximize the profitability of the transition cow.

Energy and protein requirements of the immune system

We believe the negative energy and protein balances that exist during early lactation contribute to impaired immune function and, thus, account for a portion of the periparturient immunosuppression observed. For example, the activities of neutrophils in combating infection are complex and involve expenditure of cellular energy. The average cow has ~3,500 neutrophils per μl of blood, this translates into ~1.4×10^{11} neutrophils in an 800 kg Holstein cow. The circulating half-life of neutrophils is about 6 hours, so a cow is replacing half of her neutrophils every 6 hours

from bone marrow stores. Clearly, a component of dietary energy and protein consumption for maintenance is spent on replenishment of neutrophils by the bone marrow. Conclusive evidence that the demands of lactation contribute to postpartum immune suppression derives from recent studies at the NADC. In studies with mastectomized cows it was found that they recover from periparturient immunosuppression within one week after calving, whereas intact lactating cows can be immunosuppressed for 2-3 weeks postpartum (Kimura *et al*., 1997).

The energy and protein requirements of the immune system are difficult to establish and are not well characterized in cattle. Klasing and colleagues have used isotope-labelled amino acids to study protein metabolism demands of acute phase responses in chickens (Barnes *et al*., 2002). In their studies they focused on acute phase protein synthesis by the liver with the view that the liver is a key organ in regulation of protein and amino acid metabolism. Examples of hepatic output of acute phase proteins during an immune response include a 50% increase in ceruloplasmin and a several hundred-fold increase in C-reactive protein synthesis. These may well represent a repartitioning of amino acids from skeletal muscles as a part of cachexia associated with infectious disease. Although clear experimental numbers are not available for the bovine, estimates of the daily maintenance energy and protein demands of a healthy host immune system have been predicted to be low (Klasing, 1998). However, disease conditions can rapidly cause a repartitioning of nutrient demands to the immune response and if this repartitioning response fails in a transition cow the immune response would predictably fall short of an optimal reaction.

As evidence of some of the protein requirements of the immune system, we wish to illustrate some recent findings on the leukocyte proteome and gene expression. Recent advances in molecular biology research have allowed scientists to begin the process of characterizing global levels of gene expression in circulating leukocytes from normal, uninfected cattle. Using serial analysis of gene expression (SAGE), a powerful sequence-based method to measure global gene expression levels, normal gene expression levels are now being determined for various bovine leukocyte populations. Preliminary analysis of the data indicates that selected leukocyte populations ($CD4^+$, $CD8^+$ and γδ T cells, as well as, ileal Peyer's patch follicular B cells) are metabolically active based on the high level of expression of mitochondrial, energy metabolism, biosynthetic pathways and housekeeping genes (J.D. Neill and J.F. Ridpath, NADC-USDA-ARS, unpublished). Additionally, transcripts encoding proteins necessary for the function of particular cell populations have been found. These include B and T cell receptor signalling pathway components and their regulatory proteins, proteins constituting other intracellular signalling pathways, cytokines and their cognate receptors, and other cell surface proteins. Microarray analysis of gene expression in blood neutrophils from periparturient cows has also revealed extensive changes in gene expression that affect the immune status of the transition cow (Madsen *et al*., 2004).

Recently, we have exploited advances in mass spectrometry that will enable our efforts to elucidate the bovine neutrophil proteome (J.D. Lippolis and T.A. Reinhardt, 2004, NADC-USDA-ARS, unpublished data). The summation of all data sets resulted in greater than 1400 unique peptide spectra, of which, approximately 1100 peptide spectra corresponded to unique peptide sequences. The peptide spectra were then used to match proteins from the protein database (UniREF100) and allowed them to identify greater than 250 proteins, representing the abundant proteins in circulating bovine neutrophils. The main limitation to proteomic research in dairy cows is the

incomplete nature of the genomic data. Proteomic analysis requires matching peptide spectra to potential peptides from known proteins. Just over 40% of the proteins identified matched bovine proteins in the database. The remaining ~60% identified were proteins homologous to either human, mouse or rat. The full power of proteomic research in dairy cows will be realized only upon completion of the bovine genome.

Lippolis and Reinhardt were able to group proteins in the neutrophils proteome into basic functional groups. Nearly 35% of the peptides identified were enzymes involved in basic cellular metabolic pathways. Proteins involved in cell structure/mobility or immune functions made up the next two most abundant groups of peptides with approximately 15% of the total peptide spectra each. Since circulating neutrophils have such a short half-life and a singular purpose, it should not be surprising that so much of the proteome should be dedicated to a few general functional groups. As circulating neutrophils are poised to respond to inflammation very quickly, it is reasonable that metabolic enzymes required for the primary functions of the neutrophil would be abundant. Consistent with that hypothesis, the majority of the enzymes involved in the pentose phosphate pathway and gluconeogenesis, required for NADPH generation critical for neutrophil oxidative burst, were identified. In addition, most of the enzymes involved in glycolysis and the generation of ATP were found, which would provide the cellular energy necessary for transmigration into inflamed tissue. Thus it appears that the proteome of the circulating neutrophil, is in essence in a "hair trigger" mode for eliciting a respiratory burst of oxidative metabolism to support phagocytosis.

Mass spectrometry, gene expression microarrays and SAGE are methods that allow for the survey of the genes and proteins expressed in cells or tissues. Thus far, scientists have been able to use these types of analyses to describe the basal gene and protein expression levels in normal cell populations. However, the possibility of applying these powerful approaches to understanding differences in global gene and protein expression levels in leukocytes of animals experiencing negative energy and protein balances may enable elucidation of the actual molecular basis for periparturient immunosuppression and what role the energy and protein status of the cow plays in the immune dysfunction. Proteomics may be particularly valuable for the study of neutrophils. Neutrophils need to react quickly with limited time for induction of gene expression. The lymphocyte populations, however, tend to exist predominantly in a resting state and when activated undergo considerable changes associated with an adaptive immune response where long-lived, antigen specific memory cells are an eventual outcome depending on considerable changes in gene expression and post-translational modifications.

Influence energy and protein status on immune function

Cows with ketosis, fatty liver and elevated NEFA's are believed to have poorer immune function. In vitro studies of the effects of elevated NEFA levels on selected aspects of leukocyte activities have found DNA synthesis, IgM secretion and secretion of IFN-γ to be diminished at concentrations of NEFAs often seen in serum of periparturient dairy cows (Lacetera *et al.*, 2004). These authors concluded that increases of plasma NEFA might contribute to periparturient immunosuppression and the higher incidence of postpartum infections observed in cows with a negative energy balance.

The individual and combined effects of ketones, acetate and glucose on polyclonal and antigen-specific IgM secretion by bovine blood lymphocytes have been evaluated in vitro (Nonnecke *et al*., 1992). Results indicate that plasma glucose levels associated with ketosis (1.66 *mM*) when compared with normal plasma levels (3.33 *mM*) do not affect total or antigen-specific IgM secretion by peripheral blood lymphocytes. Supplementation of cultures containing up to 3.33 *mM* glucose with ketones and/or acetate either had no effect or a modest stimulatory effect. Overall, these data indicate effects of ketones and acetate on IgM secretion are dependent on the concentration of glucose in culture, and suggest that plasma glucose, ketone and acetate concentrations associated with bovine ketosis do not alter IgM secretion. Similarly, in another study (Franklin *et al*., 1991), age-matched heifers were used to determine the effects of ketones, acetate, and glucose on mitogen-induced lymphocyte proliferation. When effects of glucose alone were evaluated, it was clear that proliferative responses of cells in medium containing glucose at 1.4 *mM* (typical of a ketotic cow) and 3.1 *mM* (typical non-ketotic cow) exceeded responses of cells in commercially available RPMI medium containing 11.1 *mM* of glucose. When culture medium was supplemented with ketones (β-hydroxybutryrate, 6.3 *mM*, acetoacetate, 7.4 *mM*), acetate (1.5 *mM*) and glucose at either 3.1 *mM* (physiologic concentration) or 1.4 *mM* (typical of ketotic cow) , proliferation of concanavalin A and phytohemagglutinin–P stimulated cells was not inhibited. Interestingly, cells stimulated with phytohemagglutinin–P in culture medium containing 1.4 or 3.1 *mM* of glucose performed substantially better when ketones were present. Pokeweed mitogen-induced responses; however, were inhibited by ketones. These differences may be attributable to the unique cell populations responding to each mitogen. Overall, these results suggest reduction in blood glucose concentrations during clinical ketosis does not inhibit mitogen-induced lymphocyte proliferation. The presence of ketones at this time may have no effect or may actually enhance proliferative responses of blood lymphocytes. This argues that NEFAs rather than ketones are contributing as immunosuppressive factors in transition cows.

Production practices on today's modern dairy farms have profound influences over the health and nutritional status of dairy cows. Supplementation of lactating cows with recombinant bovine somatotropin (rbST) results in an increase in milk yield and an improvement in efficiency of nutrient use. Homeorhesis is defined as a long-term control that expresses the genetic make-up of the animal and/or the animal's potential. Homeostasis is a short-term control representing the mechanisms that enable the animal to function under a range of environmental conditions. Scientists have integrated both homeorhetic and homeostatic controls of protein and body lipid turnover into mathematical models allowing for mechanistic representations of nutrient partitioning. Overall, rbST is a hormone involved in homeorhetic control of lactation i.e. the orchestration of physiological processes that allow greater synthesis of milk. Direct effects of rbST involve adaptations in a variety of tissue functions and the metabolism of carbohydrates, lipids, protein and minerals via alterations in key enzymes, intracellular signal transduction systems and tissue responses. Indirect effects are mediated by insulin-like growth factor-1 (IGF-1) at the level of the mammary gland. Specific changes include increased cellular rates of milk synthesis and enhanced maintenance of secretory cells, thereby improving lactation persistency (Bauman and Currie, 1980). In a study performed with lactating Holstein cows, Capuco *et al.* (Capuco *et al*., 2001) observed a gradual decrease in the number of mammary epithelial cells that largely accounted for the decline in milk production with advancing lactation. The net decline in cell number (approximately 50% during the entire lactation) resulted from continual apoptotic cell

death. However, accompanying the decline in mammary cell number by apoptosis was a degree of cell renewal. During the entire lactation, the number of new cells amounted to approximately 50% of the number of cells initially present. rbST increased the rate of cell renewal in the lactating mammary gland.

Escherichia coli induced mastitis promotes both apoptosis and cell proliferation (Long *et al*., 2001). The number of apoptotic cells was significantly higher in mastitic than in uninfected control tissue. Expression of Bax and interleukin-1β converting enzyme increased in mastitic tissues, whereas Bcl-2 expression did not differ significantly from the control. Induction of matrix metalloproteinase-9, stromelysin-1 and urokinase-type plasminogen activator was also observed in the mastitic tissue. Moreover, cell proliferation increased in the infected tissue (Long *et al*., 2001). Serum growth hormone is released during experimentally induced *E.coli* mastitis in periparturient cows (Burvenich *et al*., 1988, Shuster *et al*., 1995, Burvenich *et al*., 1999). The concentration of IGF-I in plasma didn't change whereas milk IGF-I significantly increased (Shuster *et al*., 1995, Hoeben *et al*., 1999a). An increase in IGF-I in milk has also been observed during *S. uberis* mastitis (Hoeben *et al*., 1999a). This increase is probably due to leakage of plasma IGF-I into the mammary gland and to 'de novo' synthesis in mammary epithelial cells. The local production of IGF by the mammary gland might be considered as a homeostatic mechanism that would serve to overcome extensive apoptosis.

Vandeputte-Van Messom and Burvenich treated cows with rbST before (pre-infection supplementation) and after (postinfection supplementation) experimentally induced *E. coli* mastitis (Vandeputte-Van Messom and Burvenich, 1993). This study showed that the severity of disease was significantly decreased and that rbST had beneficial effects on minimizing milk production losses and compositional changes following intramammary infection. Beneficial effects may have been mediated by the effect of IGF-I and rbST on neutrophil function. In vitro incubation of bovine PMN with rbST or IGF-I had no effect on the respiratory burst capacity of these cells. In contrast, treatment of healthy cows with rbST for 10 days stimulated neutrophil ROS production after 5 to 8 days of treatment (Burvenich *et al*., 1999). Priming effects and stimulating effects of IGF-I and somatotropin on ROS production of neutrophils and monocytes, and stimulatory effects on bone marrow have been reported earlier(Edwards *et al*., 1992, Fu *et al*., 1994). In human and rodent cells, somatotropin and IGF-I stimulated the differentiation and proliferation of granulocytic and monocytic lineages (Merchav *et al*., 1988, Scheven and Hamilton, 1991, Merchav *et al*., 1993, Merchav, 1998). The generation of ROS, phagocytosis, chemotaxis, random migration, expression of adhesion receptors and PMA induced ROS production is enhanced by IGF-I and somatotropin (Edwards *et al*., 1988, Wiedermann *et al*., 1993, Bjerknes and Aarskog, 1995, Warwick-Davies *et al*., 1995). Priming of the ROS production by neutrophils and macrophages by somatotropin and IGF-I has also been reported (Kelley, 1989, Fu *et al*., 1991, Balteskard *et al*., 1998). Apoptosis of neutrophils and promyeloid cells is prevented by IGF-I (Liu *et al*., 1997).

Hoeben *et al.* tested the effect of rbST on experimentally induced *S. uberis* mastitis (Hoeben *et al*., 1999a, Hoeben *et al*., 1999b). Beneficial effects of rbST were again observed in these cows. However, in contrast to the *E.coli* study, the positive effects were probably not due to enhancement of neutrophil function. The positive effect of rbST was probably due to a protective effect on

the blood-milk barrier. Positive effects of IGF-I on the cytoskeleton of the epithelial cells and on the tight junctions between these cells may have been involved. Indeed, effects of IGF-I or somatotropin on the cytoskeleton, tubulin mRNA, and cytoskeletal reorganization have been reported in rats (Berfield *et al.*, 1997, Goh *et al.*, 1997) and humans (Kadowaki *et al.*, 1986, Wang *et al.*, 1992). Treatment of healthy cows with rbST also protected cows from excessive milk production losses and excessive milk compositional changes during a subsequent experimental intramammary infection with *S. uberis*. Although not complete, the recovery of milk production and milk composition was markedly improved by pre-infection and postinfection supplementation with rbST.

Conclusions

Giesecke suggested that lactating dairy cows are unique in their response to stress, since ruminant metabolism is dependent on gluconeogenesis and lipogenesis/lipolysis for energy-efficient and glucose-sparing feed conversion (Giesecke, 1985). The lactation ability of dairy cows, combined with ruminant metabolism, may be a metabolically demanding phenomenon unique to dairy cows. The ensuing negative energy and protein balances in early lactation may limit the capacity of the transition cow's immune system. Periparturient immuno-suppression is likely the result of several factors working in concert with profound effects on the function of many systems of the cow. The most economically important effects of periparturient immunosuppression, are the clinical infections of the mammary gland and uterus. The best we can do today is to give periparturient cows the best possible hygiene conditions, appropriate diets and as few additional stress events as possible. Early detection and intervention of health problems in transition cows is paramount to maintaining her as an economically viable production unit in a herd.

References

Ametaj, B.N., D.C. Beitz, T.A Reinhardt. & B.J. Nonnecke (1996). 1,25-Dihydroxyvitamin D_3 inhibits secretion of interferon-γ by mitogen- and antigen-stimulated bovine mononuclear leukocytes. Vet. Immunol. Immunopathol. 52, 77-90.

Ametaj, B.N., B.J. Nonnecke, R.L. Horst & D.C. Beitz (2000). Effects of retinoic acid and 1,25-dihydroxyvitamin D_3 on IFN-γ secretion by mononuclear leukocytes from nulliparous and postparturient dairy cattle. Int. J. Vitam. Nutr. Res. 70, 92-101.

Balteskard, L., K. Unneberg, D. Halvorsen, J.B. Hansen & A. Revhaug (1998). Effects of insulin-like growth factor 1 on neutrophil and monocyte functions in normal and septic states. JPEN J Parenter Enteral Nutr 22, 127-135.

Barnes, D.M., Z. Song, K.C. Klasing & W. Bottje (2002). Protein metabolism during an acute phase response in chickens. Amino Acids 22, 15-26.

Bauman, D.E. & W.B. Currie (1980). Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. J. Dairy Sci. 63, 1514-1529.

Berfield, A.K., D. Spicer & C.K. Abrass (1997). Insulin-like growth factor I (IGF-I) induces unique effects in the cytoskeleton of cultured rat glomerular mesangial cells. J Histochem Cytochem 45, 583-593.

Bernabucci, U., B. Ronchi, L. Basirico, D. Pirazzi, F. Rueca, N. Lacetera & A. Nardone (2004). Abundance of mRNA of apolipoprotein B100, apolipoprotein E, and microsomal triglyceride transfer protein in liver from periparturient dairy cows. J. Dairy Sci. 87, 2881-2888.

Bjerknes, R. & D. Aarskog (1995). Priming of human polymorphonuclear neutrophilic leukocytes by insulin-like growth factor I: increased phagocytic capacity, complement receptor expression, degranulation, and oxidative burst. J Clin Endocrinol Metab 80, 1948-1955.

Bobe, G., J.W. Young & D.C. Beitz (2004). Invited Review: Pathology, Etiology, Prevention, and Treatment of Fatty Liver in Dairy Cows. J. Dairy Sci. 87, 3105-3124.

Burton, J.L. & M.E. Kehrli, Jr. (1995). Regulation of neutrophil adhesion molecules and shedding of *Staphylococcus aureus* in milk of cortisol- and dexamethasone-treated cows. Am. J. Vet. Res. 56, 997-1006.

Burton, J.L., M.E. Kehrli, Jr., S. Kapil & R.L. Horst (1995). Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. J. Leukocyte Biol. 57, 317-325.

Burvenich, C., E. Monfardini, J. Mehrzad, A.V. Capuco & M.J. Paape (2004). Role of neutrophil polymorphonuclear leukocytes during bovine coliform mastitis: physiology or pathology? Verh K Acad Geneeskd Belg 66, 97-153.

Burvenich, C., M.J. Paape, A.W. Hill, A.J. Guidry, R.H. Miller, R. Heyneman, W.D.J. Kremer & A. Brand (1994). Role of the neutrophil leukocyte in the local and systemic reactions during experimentally induced *E. coli* mastitis in cows immediately after calving. Vet. Q. 16, 45-50.

Burvenich, C., M.J. Paape, D. Hoeben, H. Dosogne, A.M. Massart-Leen & J. Blum (1999). Modulation of the inflammatory reaction and neutrophil defense of the bovine lactating mammary gland by growth hormone. Domest. Anim. Endocrinol. 17, 149-159.

Burvenich, C., V. Van Merris, J. Mehrzad , A. Diez-Fraile & L. Duchateau (2003). Severity of *E. coli* mastitis is mainly determined by cow factors. Vet Res 34, 521-564.

Burvenich, C., G.Vandeputte-Van Messom, E. Roets, A. Massart-Leën, R.M. Akers, R. Reynaert-Van Sichem & L. Devrieze (1988). Bovine growth hormone on acute mastitis due to Escherichia coli. Ann. Med. Vet. 132, 601-606.

Cai, T.-Q., P.G. Weston, L.A. Lund, B. Brodie, D.J. McKenna & W.C. Wagner (1994). Association between neutrophil functions and periparturient disorders in cows. Am. J. Vet. Res. 55, 934-943.

Capuco, A.V., D.L. Wood, R. Baldwin, K. McLeod & M.J. Paape, (2001). Mammary cell number, proliferation, and apoptosis during a bovine lactation: relation to milk production and effect of bST. J. Dairy Sci. 84, 2177-2187.

Daynes, R.A., E.Y. Enioutina, S. Butler, H.-H. Mu, Z.A. McGee & B.A. Araneo (1996). Induction of common mucosal immunity by hormonally immunomodulated peripheral immunization. Infect. Immun. 64, 1100-1109.

Detilleux, J.C., M.E. Kehrli, Jr., J.R. Stabel, A.E. Freeman & D.H. Kelley (1995). Study of immunological dysfunction in periparturient Holstein cattle selected for high and average milk production. Vet. Immunol. Immunopathol. 44, 251-267.

Detilleux, J.C., K.J. Koehler, A.E. Freeman, M.E. Kehrli, Jr. & D.H. Kelley 1994. Immunological parameters of periparturient Holstein cattle: genetic variation. J. Dairy Sci. 77, 2640-2650.

Dosogne, H., C. Burvenich, A.E. Freeman, M.E. Kehrli, Jr., J.C. Detilleux, J. Sulon, J.-F. Beckers & D. Hoeben (1999). Association between pregnancy-associated glycoprotein and decreased polymorphonuclear leukocyte function in early postpartum dairy cows. Vet. Immunol. Immunopathol. 67, 47-54.

Dosogne, H., A.V. Capuco, M.J. Paape, E. Roets, C. Burvenich & B. Fenwick (1998). Reduction of acyloxyacyl hydrolase activity in circulating neutrophils from cows after parturition. J. Dairy Sci. 81, 672-677.

Drackley, J.K. (1999). ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J. Dairy Sci. 82, 2259-2273.

Drackley, J.K., T.R. Overton & G.N. Douglas (2001). Adaptations of glucose and long-chain fatty acid metabolism in liver of dairy cows during the periparturient period. J. Dairy Sci. 84, E1-E13.

Edwards, C.K., 3rd, S.M. Ghiasuddin, J.M. Schepper, L.M. Yunger & K.W. Kelley (1988). A newly defined property of somatotropin: priming of macrophages for production of superoxide anion. Science 239, 769-771.

Edwards, C.K., 3rd, S.M. Ghiasuddin, L.M. Yunger, R.M. Lorence, S. Arkins, R. Dantzer & K.W. Kelley (1992). In vivo administration of recombinant growth hormone or γ-interferon activities macrophages: enhanced resistance to experimental *Salmonella typhimurium* infection is correlated with generation of reactive oxygen intermediates. Infect. Immun. 60, 2514-2521.

Franklin, S.T., J.W. Young & B.J. Nonnecke (1991). Effects of ketones, acetate, butyrate, and glucose on bovine lymphocyte proliferation. J. Dairy Sci. 74, 2507-2514.

Fu, Y.K., S. Arkins, Y.M. Li, R. Dantzer & K.W. Kelley (1994). Reduction in superoxide anion secretion and bactericidal activity of neutrophils from aged rats: reversal by the combination of gamma interferon and growth hormone. Infect. Immun. 62, 1-8.

Fu, Y.K., S. Arkins, B.S. Wang & K.W. Kelley (1991). A novel role of growth hormone and insulin-like growth factor-I. Priming neutrophils for superoxide anion secretion. J. Immunol. 146, 1602-1608.

Giesecke, W.H. (1985). The effect of stress on udder health of dairy cows. Onderstepoort J. Vet. Res. 52, 175-193.

Goff, J.P., K. Kimura & R.L. Horst (2002). Effect of mastectomy on milk fever, energy, and vitamins A, E, and beta-carotene status at parturition. J. Dairy Sci. 85, 1427-1436.

Goh, E.L., T.J. Pircher, T.J. Wood, G. Norstedt, R. Graichen & P.E. Lobie (1997). Growth hormone-induced reorganization of the actin cytoskeleton is not required for STAT5 (signal transducer and activator of transcription-5)-mediated transcription. Endocrinol. 138, 3207-3215.

Gruffat-Mouty, D., B. Graulet, D. Durand, M.E. Samson-Bouma & D. Bauchart (1999). Apolipoprotein B production and very low density lipoprotein secretion by calf liver slices. J Biochem (Tokyo) 126, 188-193.

Guidry, A.J., M.J. Paape & R.E. Pearson (1976). Effects of parturition and lactation on blood and milk cell concentrations, corticosteroids and neutrophil phagocytosis in the cow. Am. J. Vet. Res. 37, 1195-1200.

Heyneman, R., C. Burvenich & R. Vercauteren (1990). Interaction between the respiratory burst activity of neutrophil leukocytes and experimentally induced *Escherichia coli* mastitis in cows. J. Dairy Sci. 73, 985-994.

Hill, A.W., 1981. Factors influencing the outcome of *Escherichia coli* mastitis in the dairy cow. Res. Vet. Sci. 31, 107-112.

Hill, A.W., A.L. Shears & K.G. Hibbitt (1979). The pathogenesis of experimental *Escherichia coli* mastitis in newly calved dairy cows. Res. Vet. Sci. 26, 97-101.

Hippen, A.R., 2000. Glucagon as a potential therapy for ketosis and fatty liver. Vet Clin North Am Food Anim Pract 16, 267-282.

Hippen, A.R., P. She, J.W. Young, D.C. Beitz, G.L. Lindberg, L.F. Richardson & R.W. Tucker (1999a). Alleviation of fatty liver in dairy cows with 14-day intravenous infusions of glucagon. J. Dairy Sci. 82, 1139-1152.

Hippen, A.R., P. She, J.W. Young, D.C. Beitz, G.L. Lindberg, L.F. Richardson & R.W. Tucker (1999b). Metabolic responses of dairy cows and heifers to various intravenous dosages of glucagon. J. Dairy Sci. 82, 1128-1138.

Hirvonen, J., K. Eklund, A.M. Teppo, G. Huszenicza, M. Kulcsar, H. Saloniemi & S. Pyorala (1999). Acute phase response in dairy cows with experimentally induced Escherichia coli mastitis. Acta Vet. Scand. 40, 35-46.

Hocquette, J.F. & D. Bauchart (1999). Intestinal absorption, blood transport and hepatic and muscle metabolism of fatty acids in preruminant and ruminant animals. Reprod. Nutr. Dev. 39, 27-48.

Hoeben, D., C. Burvenich, P.J. Eppard, J.C. Byatt & D.L. Hard (1999a). Effect of bovine somatotropin on neutrophil functions and clinical symptoms during *Streptococcus uberis* mastitis. J. Dairy Sci. 82, 1465-1481.

Hoeben, D., C. Burvenich, P.J. Eppard & D.L. Hard (1999b). Effect of recombinant bovine somatotropin on milk production and composition of cows with *Streptococcus uberis* mastitis. J. Dairy Sci. 82, 1671-1683.

Hogan, J.S., K.L. Smith, K.H. Hoblet, P.S. Schoenberger, D.A. Todhunter, W.D. Hueston, D.E. Pritchard, G.L. Bowman, L.E. Heider, B.L. Brockett & H.R. Conard (1989). Field survey of clinical mastitis in low somatic cell count herds. J. Dairy Sci. 72, 1547-1556.

Ishikawa, H., T. Shirahata & K. Hasegawa (1994). Interferon-γ production of mitogen stimulated peripheral lymphocytes in perinatal cows. J Vet Med Sci 56, 735-738.

Kadowaki, T., S. Koyasu, E. Nishida, H. Sakai, F. Takaku, I. Yahara & M. Kasuga (1986). Insulin-like growth factors, insulin, and epidermal growth factor cause rapid cytoskeletal reorganization in KB cells. Clarification of the roles of type I insulin-like growth factor receptors and insulin receptors. J. Biol. Chem. 261, 16141-16147.

Katoh, N. (2002). Relevance of apolipoproteins in the development of fatty liver and fatty liver-related peripartum diseases in dairy cows. J Vet Med Sci 64, 293-307.

Kehrli, M.E., Jr. & J.P. Goff (1989). Periparturient hypocalcemia in cows: effects on peripheral blood neutrophil and lymphocyte function. J. Dairy Sci. 72, 1188-1196.

Kehrli, M.E., Jr., B.J. Nonnecke & J.A. Roth (1989a). Alterations in bovine lymphocyte function during the periparturient period. Am. J. Vet. Res. 50, 215-220.

Kehrli, M.E., Jr., B.J. Nonnecke & J.A. Roth (1989b). Alterations in bovine neutrophil function during the periparturient period. Am. J. Vet. Res. 50, 207-214.

Kehrli, M.E., Jr., K.A. Weigel, A.E. Freeman, J.R. Thurston & D.H. Kelley (1991). Bovine sire effects on daughters' in vitro blood neutrophil functions, lymphocyte blastogenesis, serum complement and conglutinin levels. Vet. Immunol. Immunopathol. 27, 303-319.

Kelley, K.W. (1989). Growth hormone, lymphocytes and macrophages. Biochem Pharmacol 38, 705-713.

Kimura, K., J.P. Goff & M.E. Kehrli, Jr. (1999). Effects of the presence of the mammary gland on expression of neutrophil adhesion molecules and myeloperoxidase activity in periparturient dairy cows. J. Dairy Sci. 82, 2385-2392.

Kimura, K., J.P. Goff, M.E. Kehrli, Jr., J.A. Harp & B.J. Nonnecke (1997). Effect of mastectomy on phenotype and function of leukocytes in periparturient dairy cows. J. Dairy Sci. 80, 264.

Kimura, K., J.P. Goff, M.E. Kehrli, Jr., J.A. Harp & B.J. Nonnecke (2002). Effects of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows. J. Dairy Sci. 85, 1437-1444.

Klasing, K.C., 1998. Nutritional modulation of resistance to infectious diseases. Poult. Sci. 77, 1119-1125.

Kornalijnslijper, E., B. Beerda, I. Daemen, J. van der Werf, T. van Werven, T. Niewold, V. Rutten & E. Noordhuizen-Stassen (2003). The effect of milk production level on host resistance of dairy cows, as assessed by the severity of experimental *Escherichia coli* mastitis. Vet Res 34, 721-736.

Lacetera, N., D. Scalia, O. Franci, U. Bernabucci, B. Ronchi & A. Nardone (2004). Short communication: effects of nonesterified fatty acids on lymphocyte function in dairy heifers. J. Dairy Sci. 87, 1012-1004.

Lee, E.-K. & M.E. Kehrli Jr. (1998). Expression of adhesion molecules on neutrophils of periparturient cows and neonatal calves. Am. J. Vet. Res. 59, 37-43.

Lie, O., H. Solbu & M. Syed (1986). A genetic association between bovine serum and colostrum lysozyme levels. Anim. Genet. 17, 39-45.

Lie, O., M. Syed & H. Solbu (1983). The genetic influence on serum haemolytic complement levels in cattle. Anim. Blood Groups Biochem. Genet. 14, 51-57.

Liu, Q., D. Schacher, C. Hurth, G.G. Freund, R. Dantzer & K.W. Kelley (1997). Activation of phosphatidylinositol 3'-kinase by insulin-like growth factor-I rescues promyeloid cells from apoptosis and permits their differentiation into granulocytes. J. Immunol. 159, 829-837.

Long, E., A.V. Capuco, D.L. Wood, T. Sonstegard, G. Tomita, M.J. Paape & X. Zhao (2001). *Escherichia coli* induces apoptosis and proliferation of mammary cells. Cell Death Differ 8, 808-816.

Madsen, S.A., L.C. Chang, M.C. Hickey, G.J. Rosa, P.M. Coussens & J.L. Burton (2004). Microarray analysis of gene expression in blood neutrophils of parturient cows. Physiol Genomics 16, 212-221.

Malinowski, E., J. Krzyzanowski, W. Wawron, J. Slawomirski & J. Gluszak (1983). Analysis of cases of *Escherichia coli* mastitis in cows. Med. Weter. 39, 608-610.

Mazur, A., M. Ayrault-Jarrier, Y. Chilliard & Y. Rayssiguier (1992). Lipoprotein metabolism in fatty liver dairy cows. Diabete Metab 18, 145-149.

McDermott, C.M., J.S. Cullor & B.W. Fenwick (1991a). Intracellular and extracellular enzymatic deacylation of bacterial endotoxin during localized inflammation induced by *Escherichia coli*. Infect. Immun. 59, 478-485.

McDermott, C.M., J.L. Morrill & B.W. Fenwick (1991b). Deacylation of endotoxin during natural cases of bovine mastitis. J. Dairy Sci. 74, 1227-1234.

Mehrzad, J., H. Dosogne, E. Meyer, R. Heyneman & C. Burvenich (2001). Respiratory burst activity of blood and milk neutrophils in dairy cows during different stages of lactation. J. Dairy Res. 68, 399-415.

Mehrzad, J., L. Duchateau, S. Pyorala & C. Burvenich (2002). Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. J. Dairy Sci. 85, 3268-3276.

Merchav, S. (1998). The haematopoietic effects of growth hormone and insulin-like growth factor-I. J Pediatr Endocrinol Metab 11, 677-685.

Merchav, S., M. Lake & A. Skottner (1993). Comparative studies of the granulopoietic enhancing effects of biosynthetic human insulin-like growth factors I and II. J Cell Physiol 157, 178-183.

Merchav, S., I. Tatarsky & Z. Hochberg (1988). Enhancement of human granulopoiesis in vitro by biosynthetic insulin-like growth factor I/somatomedin C and human growth hormone. J. Clin. Invest. 81, 791-797.

Monfardini, E., M.J. Paape, Y. Wang, A.V. Capuco, M. Husheem, L. Wood & C. Burvenich (2002). Evaluation of L-selectin expression and assessment of protein tyrosine phosphorylation in bovine polymorphonuclear neutrophil leukocytes around parturition. Vet Res 33, 271-281.

Newbould, F.H.S. (1976). Phagocytic activity of bovine leukocytes during pregnancy. Can. J. Comp. Med. 40, 111-116.

Nonnecke, B.J., S.T. Franklin & J.W. Young (1992). Effects of ketones, acetate, and glucose on in vitro immunoglobulin secretion by bovine lymphocytes. J. Dairy Sci. 75, 982-990.

Nonnecke, B.J., K. Kimura, J.P. Goff & M.E. Kehrli Jr. (2003). Effects of the mammary gland on functional capacities of blood mononuclear leukocyte populations from periparturient cows. J. Dairy Sci. 86, 2359-2368.

Scheven, B.A. & N.J. Hamilton (1991). Stimulation of macrophage growth and multinucleated cell formation in rat bone marrow cultures by insulin-like growth factor I. Biochem. Biophys. Res. Commun. 174, 647-653.

Shafer-Weaver, K.A., G.M. Pighetti & L.M. Sordillo (1996). Diminished mammary gland lymphocyte functions parallel shifts in trafficking patterns during the postpartum period. Proc. Soc. Exp. Biol. Med. 212, 271-279.

Shafer-Weaver, K.A. & L.M. Sordillo (1997). Bovine CD8+ suppressor lymphocytes alter immune responsiveness during the postpartum period. Vet. Immunol. Immunopathol. 56, 53-64.

She, P., A.R. Hippen, J.W. Young, G.L. Lindberg, D.C. Beitz, L.F. Richardson & R.W. Tucker (1999). Metabolic responses of lactating dairy cows to 14-day intravenous infusions of glucagon. J. Dairy Sci. 82, 1118-1127.

Shuster, D.E., M.E. Kehrli, Jr. & C.R. Baumrucker (1995). Relationship of inflammatory cytokines, growth hormone, and insulin-like growth factor-I to reduced performance during infectious disease. Proc. Soc. Exp. Biol. Med. 210, 140-149.

Shuster, D.E., E.-K. Lee & M.E. Kehrli, Jr. (1996). Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in periparturient versus midlactation cows. Am. J. Vet. Res. 57, 1569-1575.

Smith, K.L., D.A. Todhunter & P.S. Schoenberger (1985a). Environmental mastitis: cause, prevalence, prevention. J. Dairy Sci. 68, 1531-1553.

Smith, K.L., D.A. Todhunter & P.S. Schoenberger (1985b). Environmental pathogens and intramammary infection during the dry period. J. Dairy Sci. 68, 402-417.

Sordillo, L.M., M.J. Redmond, M. Campos, L. Warren & L.A. Babiuk (1991). Cytokine activity in bovine mammary gland secretions during the periparturient period. Can. J. Vet. Res. 55, 298-301.

Stabel, J.R., J.P. Goff & K. Kimura (2003). Effects of supplemental energy on metabolic and immune measurements in periparturient dairy cows with Johne's disease. J. Dairy Sci. 86, 3527-3535.

Van Werven, T., E.N. Noordhuizen-Stassen, A.J. Daemen, Y.H. Schukken, A. Brand & C. Burvenich (1997). Preinfection in vitro chemotaxis, phagocytosis, oxidative burst, and expression of CD11/CD18 receptors and their predictive capacity on the outcome of mastitis induced in dairy cows with *Escherichia coli*. J. Dairy Sci. 80, 67-74.

Vandeputte-Van Messom, G., C. Burvenich, E. Roets, A.M. Massart-Leen, R. Heyneman, W.D. Kremer & A. Brand (1993). Classification of newly calved cows into moderate and severe responders to experimentally induced *Escherichia coli* mastitis. J. Dairy Res. 60, 19-29.

Vandeputte-Van Messom, G.V. & C. Burvenich (1993). Effect of somatotropin on changes in milk production and composition during coliform mastitis in periparturient cows. J. Dairy Sci. 76, 3727-3741.

Vangroenweghe, F., L. Duchateau & C. Burvenich (2004). Moderate inflammatory reaction during experimental *Escherichia coli* mastitis in primiparous cows. J. Dairy Sci. 87, 886-895.

Van der Valk, P. & C.J. Herman (1987). Leukocyte functions. Lab. Invest. 56:127-37.

Wang, C., Y. Li, B. Wible, K.J. Angelides & D.N. Ishii (1992). Effects of insulin and insulin-like growth factors on neurofilament mRNA and tubulin mRNA content in human neuroblastoma SH-SY5Y cells. Brain Res Mol Brain Res 13, 289-300.

Warwick-Davies, J., D.B. Lowrie & P.J. Cole (1995). Growth hormone is a human macrophage activating factor. Priming of human monocytes for enhanced release of H2O2. J. Immunol. 154, 1909-1918.

Weber, P.S., S.A. Madsen, G.W. Smith, J.J. Ireland & J.L. Burton (2001). Pre-translational regulation of neutrophil L-selectin in glucocorticoid- challenged cattle. Vet. Immunol. Immunopathol. 83, 213-240.

Weber, P.S., Toelboell, T., Chang, L.C., Tirrell, J.D., Saama, P.M., Smith, G.W. & Burton, J.L., (2004). Mechanisms of glucocorticoid-induced down-regulation of neutrophil L-selectin in cattle: evidence for effects at the gene-expression level and primarily on blood neutrophils. J Leukoc Biol 75, 815-827.

Weigel, K.A., M.E. Kehrli, Jr., A.E. Freeman, J.R. Thurston, M.J. Stear & D.H. Kelley (1991). Association of class I bovine lymphocyte antigen complex alleles with in vitro blood neutrophil functions, lymphocyte blastogenesis, serum complement and conglutinin levels in dairy cattle. Vet. Immunol. Immunopathol. 27, 321-335.

Wells, P.W., C. Burrells & W.B. Martin (1977). Reduced mitogenic responses in cultures of lymphocytes from newly calved cows. Clin. Exp. Immunol. 29, 159-161.

Wiedermann, C.J., N. Reinisch & H. Braunsteiner (1993). Stimulation of monocyte chemotaxis by human growth hormone and its deactivation by somatostatin. Blood 82, 954-960.

Yang, T.J., I.A. Ayoub & M.J. Rewinski (1997). Lactation stage-dependent changes of lymphocyte subpopulations in mammary secretions: inversion of CD4+/CD8+ T cell ratios at parturition. Am. J. Reprod. Immun. 37, 378-383.

Vitamin and trace mineral effects on immune function of ruminants

W.P. Weiss[1] and J.W. Spears[2]
[1]The Ohio State University, Department of Animal Sciences, 1680 Madison Avenue, Wooster OH 44691, USA
[2]North Carolina State University, Department of Animal Science, Raleigh OH 27965, USA

Abstract

The scientific literature is replete with studies showing that trace mineral and vitamin nutrition influence immune function in nonruminants. The data with ruminants is much more limited and generally less consistent. Supplementation of Cr often, but not always, improves cellular immune responses. The majority of data show positive effects of Cu supplementation on neutrophil function, but effects on cellular and humoral immunity are inconsistent. The preponderance of data show positive effects of Se and/or vitamin E supplementation on immune function and disease resistance. Vitamin A supplementation has not affected immune function consistently whereas B-carotene supplementation may improve neutrophil function. The effects of Zn supplementation on immune function are usually minor. The lack of demonstrative effects of altering the intake of many vitamin and minerals on immune function may mean that the immune system has a high priority for those nutrients and the nutrients provided by the basal diet are adequate to maintain immune function.

Keywords: trace minerals, vitamins, antioxidants, immunity, health

Introduction

Numerous vitamins and minerals are integral to the proper functioning of the immune system of ruminants. Alterations in immune function in response to a deficiency or to supplementation of a mineral or vitamin depend on the specific function of the nutrient and to the relative priority of the immune system for that specific nutrient. The lack of a response in immune function to decreased status of a specific vitamin or mineral should not be interpreted to imply that the nutrient is not involved with immune function.

The immune system is arguably the most complex and diverse physiological system in mammals, which makes analytical assessment of immunity difficult. Currently available in vitro and in vivo assays measure very limited aspects of immune function and do not assess the complete response of the immune system to a specific challenge. Moreover, the response of the immune system varies depending upon the infective agent and site of infection. Different measures of immune function have been related with disease in cattle (Cai *et al*., 1994; Kimura *et al*., 2002); however, Suttle and Jones (1989) cautioned against assuming that changes in immune function elicited by changing the nutritional status of an animal will affect disease resistance.

Numerous reviews are available that discuss relationships between the supply of specific vitamins and minerals and immune function in non-ruminants (Griffin *et al*., 2003; Mackenzie *et al*., 2002; Meydani and Beharka, 1998; Percival, 1998; Shankar and Prasad, 1998; Stephensen, 2001). This paper will be limited to discussing the effects of varying the supply of vitamins and minerals on immune function and disease prevalence of ruminants.

Factors affecting immune response to vitamin and mineral supply

Spears (2000) outlined several reasons why experiments evaluating the effects of altering the supply of minerals and vitamins on immune function and health of ruminants often provide inconsistent results. These include:

- Differences in nutrient status of animals;
  Because of body reserves, several months of feeding a specific diet may be required to alter the status of some trace minerals and vitamins.
- Differences in the concentrations of the nutrient in the basal diet;
  The concentration of trace minerals in feeds are extremely variable because of differences in concentrations of minerals in soil and vitamin concentrations in feeds vary because of differences in processing and storage methods (NRC, 2001).
- Differences in bioavailability of differences sources of the nutrient;
  Bioavailability of trace minerals and vitamins (usually assessed by tissue concentrations) differ among sources (Spears, 2003) and some data suggest that metabolism of trace minerals also may differ between inorganic and organic sources (Spears, 1989).
- Differences in physiological and metabolic demands for the nutrient among animals.
  Animals that are under stress or high producing animals have increased requirements for trace minerals and vitamins. Certain stresses are associated with increased excretion of certain trace minerals and decreased concentrations of some trace minerals and vitamins in blood plasma (Arthington *et al*., 1997; Erskine and Bartlett, 1993; Goff and Stabel, 1990; Nockels *et al*., 1993; Weiss *et al*., 1990).

The complexity of the immune system also contributes to inconsistencies among experiments; different minerals and vitamins could exert effects on immunity in several different ways. The best understood relationship between immune function and micronutrients is that for the antioxidant minerals and vitamins. Antioxidant nutrients have direct effects on phagocytic cell function by protecting them from reactive oxygen species produced by the respiratory burst (Figure 1). Vitamins and minerals could alter cytokine production via direct or indirect effects on gene expression and cytokines have profound effects on immune response. Different antigens or pathogens elicit distinct types of immune response which may be affected differently by vitamin and mineral nutriture. If an antigen interacts directly with B-cells, and a particular nutrient is needed by T-cells, a B-cell response to supplementation of that nutrient is unlikely. The measures of immune function used in different experiments also contribute to variability (McMurray, 1984).

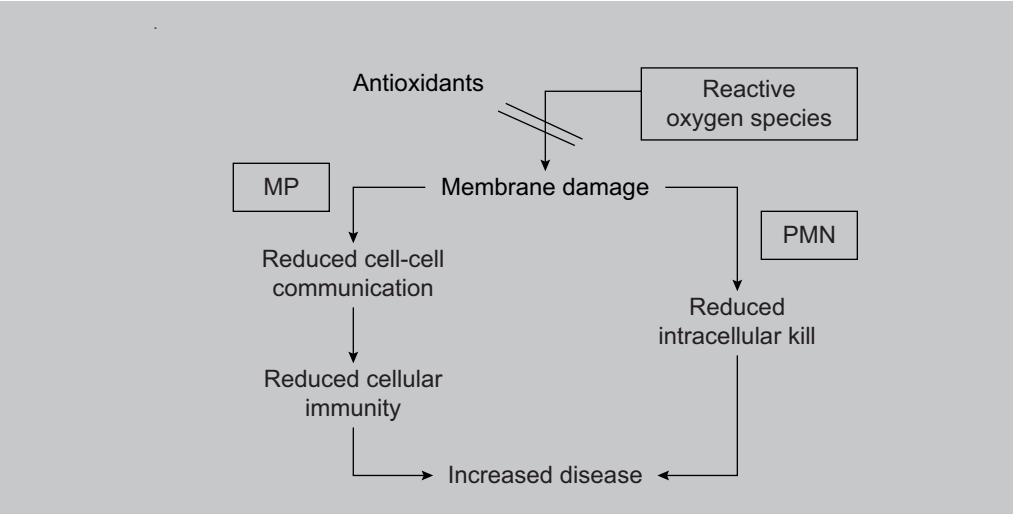

Figure 1. Possible mechanism by which reactive oxygen species (free radicals) can negatively impact immune cell function. Antioxidant nutrients can prevent initiation of the process. MP = macrophage; PMN = polymorphonucleated neutrophil.

Chromium

The best understood metabolic function of Cr is as a component of the glucose tolerance factor (GTF), which enhances the action of insulin. Chromium can increase the uptake of glucose and oxidation of glucose by peripheral tissues. If immune cells respond similarly, Cr could increase their supply of energy yielding substrate. Chromium also appears to be involved with lipid metabolism and gene regulation (some or all of these effects may be mediated via insulin) and may have antioxidant properties in vivo. Numerous studies have reported alterations in immune function when Cr was supplemented, but the mode of action remains to be elucidated (Borgs and Mallard, 1998). In ruminant studies, supplementation rates ranged from about 0.15 to 1.0 mg/kg of diet dry matter (median supplementation rate for those studies was approximately 0.5 mg/kg). The source of supplemental Cr also varied and included Cr-enriched yeast, Cr nicotinate, Cr picolinate, Cr-amino acid chelates, and $CrCl_3$.

Chromium supplementation does not appear to affect phagocytic or killing ability of bovine neutrophils. Neutrophils isolated from dairy cows that were fed control diets or diets with 0.25 to 0.5 mg/kg supplemental Cr had similar phagocytic activity (Chang *et al.*, 1996a; Faldyna *et al.*, 2003). Chromium supplementation did not affect oxidative burst response (an indirect measure of killing ability) in neutrophils isolated from dairy cows (Faldyna *et al.*, 2003), and neutrophils from calves fed supplemental Cr had similar killing ability against *Staphylococcus aureus* as those from calves fed the control diet (Arthington *et al.*, 1997). The lack of an effect of Cr on killing activity of neutrophils is not surprising. Killing ability of neutrophils is clearly influenced by antioxidants (discussed in later sections) and although Cr may have some antioxidant properties

in vivo, its concentrations in tissues is probably too low to substantially impact antioxidant status.

With growing ruminants (nine studies used cattle and one study used sheep) Cr supplementation decreased concentrations of serum or plasma cortisol in five studies, had no effect in four studies and increased concentrations in the one study using lambs (Table 1). With early lactation dairy cows, one study reported that Cr-supplementation increased cortisol and one reported decreased cortisol concentrations (Table 1). Glucocorticoids such as cortisol can be immunosuppressive. Corticosteroid administration reduces the migration of bovine blood neutrophils (Burton *et al*., 1995a), reduces T-cell function or response (Nonnecke *et al*., 1997), and has variable effects on B-cells (Nonnecke *et al*., 1997). Some of the changes in immune function observed when Cr is supplemented could be mediated by changes in cortisol concentrations.

The effect of Cr supplementation on lymphocyte blastogenesis has been variable among studies but a major source of variation appears to be the mitogen used in the assay (Table 2). Almost all studies that used concanavalin A (ConA) as the stimulant reported that Cr supplementation (either in vivo or in vitro) increased lymphocyte blastogenesis, but Cr usually had no effect when polk weed mitogen (PWM) or phytohemagglutinin (PHA) were used as the mitogen. Polk weed mitogen stimulates primarily T-helper cells and not cytotoxic T-lymphocytes (Franklin *et al*., 1994) whereas ConA and PHA stimulate cytotoxic T-lymphocytes. Delayed hypersensitivity was measured in a few experiments (Table 2) and in general, results agree with blastogenic results. The experiments that used PHA reported positive effects of Cr supplementation but the studies that used a skin contact antigen reported no effect. The results summarized in Table 2 show that T-cell function is affected by Cr supplementation, but Cr more likely affects cytotoxic T-lymphocytes rather than T-helper cells. The reason for this is not known.

Table 1. Effects of supplemental chromium on blood serum or plasma concentrations of cortisol.

Type of animal	Supplementation	Reference
Decreased concentrations		
Holstein calves (50 kg)	0.4 mg/kg	Kegley *et al*., 1996
Steers (240 kg)	0.2, 0.5, 1.0 mg/kg	Moonsie-Shageer and Mowat, 1993
Beef calves (245 kg)	0.4 mg/kg	Chang and Mowat, 1992a
Steers (233 kg)	1 mg/kg	Mowat *et al*., 1993
Steers (236 kg)	0.75 mg/kg	Chang *et al*., 1995
Early lactation dairy cows	0.5 mg/kg	Yang *et al*., 1996
No effect on concentrations		
Steers (179 kg)	4 mg/d	Lindell *et al*., 1994
Holstein calves (84 kg)	3 mg/d	Arthington *et al*., 1997
Steers (215 kg)	0.4 mg/kg	Kegley and Spears, 1995
Steers (250 kg)	0.14 mg/kg	Wright *et al*., 1994
Increased concentrations		
Lambs (50 kg)	0.4 mg/kg	Gentry *et al*., 1999
Early lactation dairy cows	0.5 mg/kg	Burton *et al*., 1995b

The effects of Cr on humoral immunity have been evaluated by measuring production of specific antibodies following administration of a foreign protein or a vaccine. The effects of Cr on antibody titers following vaccination or disease challenge have been inconsistent among and within experiments. For example, in beef feedlot calves, Cr supplementation from Cr-yeast did not affect antibody titers following vaccination against four common bovine viral diseases, and Cr from a Cr-chelate had no effect on antibody titers for three of the four vaccines (Chang *et al*., 1996b). Neither Cr source affected antibody titers following vaccination against a species of bacteria. Antibody titers against ovalbumin were increased with Cr-yeast but not affected by Cr-chelate. In another study, Cr supplementation increased antibody titers against infectious bovine rhinotracheitis (IBR) but not bovine parainfluenza virus type 3 (PI-3) following vaccination (Burton *et al*., 1994). Both Burton *et al*. (1993) and Kegley *et al*. (1997) reported no effect of Cr supplementation on antibody titers to various antigens, but Faldyna *et al*. (2003) reported that Cr supplementation increased antibody titers against tetanus toxin. Overall, across all antigens, Cr sources, and experiments, supplemental Cr increased antibody titers 4 of 19 times.

The ultimate measure of enhanced immune function is increased resistance to disease. Morbidity increases when animals are stressed. Common stress events for cattle include weaning, transporting to a feedlot (including the associated short term fast and water deprivation), introduction into a new environment with new cattle, and parturition. Chromium supplementation reduced morbidity in beef calves following transportation and/or a short term fast in several studies (Lindell *et al*., 1994; Moonsie-Shageer and Mowat, 1993; Mowat *et al*., 1993), but a similar number of studies reported no effect of Cr on morbidity (Chang *et al*., 1995; Chang and Mowat, 1992b; Mathison and Engstrom, 1995; Wright *et al*., 1994). Chromium supplementation of dairy cows had no effect on incidence of clinical mastitis or intramammary infections (Chang *et al*., 1996a). When cattle were challenged experimentally with different pathogens (*Pasteurella haemolytica* and IBR), Cr supplementation generally had few beneficial effects (Arthington *et al*., 1997; Kegley *et al*., 1996; Kegley *et al*., 1997). Kegley *et al*. (1996), however, reported transient decreases in body temperature following intranasal inoculation with IBR when beef calves were supplemented with Cr.

Copper

Copper serves as a cofactor for a myriad of enzymes that are involved with bone development, amino acid metabolism, skin pigmentation, and control of reactive oxygen species. The antioxidant functions of copper are directly related to certain immunological functions (i.e., phagocytic cell function) and disease resistance, and numerous studies have evaluated the effect of altered Cu status on neutrophil function (Table 3). Experiments have also been conducted to examine the influence of Cu status on lymphocyte function. Copper status of ruminants can be reduced by reducing the intake of Cu, by feeding Cu antagonists such as molybdenum, sulfur, and iron, or by imposing certain stresses on the animal that increase urinary excretion of Cu. The use of antagonists to influence Cu status clouds interpretation because results could be a direct cause of the antagonist or an indirect effect via reduced Cu status. Results will generally be discussed assuming that the effects observed were caused by changes in Cu status.

Neutrophils are an extremely important part of host defense and reduced neutrophil function is associated with several disorders in cattle (Cai *et al*., 1994; Hill *et al*., 1979). To provide host defense, neutrophils must first migrate to the infection site, then phagocytize the pathogen, and finally kill the pathogen. The influence of Cu status on all three activities has been evaluated with ruminants. In vivo migration of neutrophils was increased (no effect was observed on in vitro migration) when heifers were fed a Cu depleting diet (Arthington *et al*., 1996a). Changes in ceruloplasmin could account for the migration response. In a concurrent experiment with heifers fed the same diets (Cerone *et al*., 1998), the diet that increased neutrophil migration also decreased plasma ceruloplasmin concentration. Using an in vitro system, Broadley and Hoover (1989) found that increased ceruloplasmin reduced the adhesion of neutrophils to epithelial cells. Adhesion is required for neutrophils to migrate to an infection site.

The effect of Cu status on phagocytosis has been inconsistent with the majority of studies reporting no effect but some studies report decreased phagocytosis when Cu status was reduced. The data are much clearer with respect to killing ability. Eight studies were found that measured killing ability when Cu status was altered and six reported reduced killing and two reported no

Table 2. Effect of supplemental chromium on measures of on ruminant T-lymphocyte functions.

Response	Reference
Blastogenesis stimulated with concanavalin A	
Increased (based on cytokine concentrations)	Burton *et al*., 1996
Increased (Cr added to media)	Chang *et al*., 1996a
Increased (serum from cows fed Cr added to media)	Burton *et al*., 1995b
Increased	Burton *et al*., 1993
Increased	Chang *et al*., 1994
Increased (Cr added to media)	Chang *et al*., 1994
Increased	Faldyna *et al*., 2003
No effect	Arthington *et al*., 1997
Blastogenesis stimulated with polk weed mitogen	
No effect	Arthington *et al*., 1997
No effect	Kegley *et al*., 1996
No effect	Kegley and Spears, 1995
Decreased	Gentry *et al*., 1999
Blastogenesis stimulated with phytohemagglutinin	
Increased	Gentry *et al*., 1999
No effect	Arthington *et al*., 1997
No effect	Kegley *et al*., 1996
No effect	Kegley and Spears, 1995
Delayed hypersensitivity reactions	
Increased (intradermal phytohemagglutinin)	Kegley *et al*., 1996
Increased (intradermal phytohemagglutinin)	Kegley and Spears, 1995
No effect (percutaneous dinitrochlorobenzene)	Kegley *et al*., 1997
No effect (percutaneous dinitrochlorobenzene)	Moonsie-Shageer and Mowat, 1993

effect (Table 3). In addition, the magnitude of the respiratory burst in neutrophils (Cerone *et al*., 1998) and macrophages (Cerone *et al*., 2000a) is lower when Cu status is reduced. Copper is a component of the enzyme, superoxide dismutase (SOD; EC 1.15.1.1) which converts superoxide to hydrogen peroxide and is an important part of the antioxidant system of cells. Superoxide is produced by the respiratory burst and is absolutely necessary for killing; however, high superoxide concentrations are also detrimental to neutrophil cells. Activity of SOD in red blood cells and neutrophils is usually reduced when Cu status is reduced (Arthington *et al*., 1996b; Cerone *et al*., 2000b; Gengelbach *et al*., 1997; Gengelbach and Spears, 1998; Xin *et al*., 1991). The effect of Cu deficiency on SOD could account for the reduced killing ability of neutrophils.

Approximately 95% of the Cu found in plasma is in ceruloplasmin, a Cu-containing plasma protein (Hellman and Gitlin, 2002). This protein is a ferroxidase that oxidizes ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). The activity of plasma ceruloplasmin in ruminants is reduced when Cu status is poor (Cerone *et al*., 2000b; Gengelbach and Spears, 1998) and that could possibly increase concentrations of Fe^{3+} (i.e., reactive Fe) in tissues. Ferric iron participates in Fenton-type reactions that can cause substantial oxidation and peroxidation within cells and tissues. Ceruloplasmin in an in vitro system has also been shown to reduce the amount of superoxide produced by neutrophils (Broadley and Hoover, 1989). Increased oxidative stress caused by lower activity of ceruloplasmin is another potential mechanism for the effect of Cu status on neutrophil function.

Both cell-mediated and humoral immunity in rodents are greatly depressed by Cu deficiency (Prohaska and Failla, 1993), however, studies with cattle have failed to show consistent effects of

Table 3. Effect of copper status on phagocytosis and killing ability.

Response[1]	Animal/treatment[2]	Reference
Phagocytic activity		
Decreased	Beef cows; Trt = high Fe	Niederman *et al*., 1994
Decreased	Sheep; Trt = high Mo+S	Olkowski *et al*., 1990
No effect	Steers; Trt = high Mo	Xin *et al*., 1991
No effect	Calves; Trt = low Cu	Boyne and Arthur, 1981
No effect	Sheep and calves; Trt = low Cu	Jones and Suttle, 1981
Killing ability (pathogen)		
Decreased (*S. aureus*)	Calves; Trt = high Mo	Gengelbach *et al*., 1997
Decreased (*S. aureus*)	Steers; Trt = high Mo	Xin *et al*., 1991
Decreased (*C. albicans*)	Cattle; Trt = low Cu	Boyne and Arthur, 1981
Decreased (*C. albicans*)	Cattle; Trt = high Mo or high Fe	Boyne and Arthur, 1986
Decreased (*C. albicans*)	Sheep and calves; Trt = low Cu	Jones and Suttle, 1981
Decreased (*S. aureus*)	Heifers; Trt = low Cu	Torre *et al*., 1996
No effect (*S. aureus*)	Heifers; Trt = high Mo+S	Arthington *et al*., 1995
No effect (*C. albicans*)	Sheep; Trt = high Mo+S	Olkowski *et al*., 1990

[1]Effect of reducing Cu status (i.e., adequate Cu vs. reduced available Cu supply).
[2]Control diet assumed to be adequate in Cu, treatments shown was used to reduce Cu status.

Cu deficiency on either aspect of immunity. Several experiments with semi-purified diets with very low Cu concentrations and diets with added Mo found no effect of Cu status on blastogenesis or delayed-type hypersensitivity (Arthington *et al*., 1995; Arthington *et al*., 1996b; Stabel *et al*., 1993; Torre *et al*., 1996; Ward *et al*., 1993; Ward *et al*., 1997; Ward and Spears, 1999). However, in some studies, those measures of immunity were improved when Cu was supplemented to cattle with Cu deficiency (Ward *et al*., 1997; Wright *et al*., 2000). Antibody response to different antigens was improved when Cu status was improved in some studies (Gengelbach and Spears, 1998; Ward and Spears, 1999), but the majority of experiments found no effect (Niederman *et al*., 1994; Stabel *et al*., 1993; Ward *et al*., 1997). In general, currently available data do not suggest a strong relationship between Cu status and cellular and humoral immunity in cattle.

The effect of Cu status of ruminants on resistance to naturally occurring infections and experimental challenge models has been studied in only a few experiments. Copper deficiency in lambs caused by low Cu intake increased susceptibility to bacterial infection and increased mortality (Woolliams *et al*., 1986). Cattle that were adequate in Cu had a greater febrile response following a challenge to IBR or *P. Haemolytica* in two studies (Gengelbach *et al*., 1997; Stabel *et al*., 1993). Arthington *et al*. (1996b) reported a nonsignificant increased febrile response in Cu-adequate cattle following a bovine herpesvirus-1 challenge. These data suggest that Cu-adequate cattle may be better able to mount an inflammatory response than Cu-deficient cattle. Conversely, Scaletti *et al*. (2003) reported that Cu-inadequate dairy cows had a greater febrile response following an intramammary challenge with *Escherichia coli* than Cu-adequate cows. Cows in adequate Cu-status had less severe clinical signs of mastitis than Cu-inadequate cows. The reason for the reduced febrile response following an intramammary bacterial challenge is probably because *E. coli* numbers in the infected gland were lower in Cu-adequate cows. The generally improved neutrophil function (Table 3) when Cu is adequate may be the reason pathogen numbers were lower.

Selenium and vitamin E

Although selenium and vitamin E have unique biochemical functions, their functions are related and a response to supplementation of one often depends on status of the other. Vitamin E is probably the most important lipid soluble antioxidant in mammalian cells (Burton, 1994). Its primary function is to react with fatty acid radicals and stop the propagation of fatty acid peroxidation reactions. Selenium as a cofactor of different glutathione peroxidases, and perhaps other proteins, is an important antioxidant nutrient (Behne and Kyriakopoulos, 2001). The glutathione peroxidase found in the cytosol (GSHpx; EC 1.11.1.9) is involved in the conversion of hydrogen peroxide into water. Phospholipid hydroperoxide glutathione peroxidase (PH-GSHpx; EC 1.11.1.12) is associated with cellular membranes and its substrate is lipid hydroperoxides. In this capacity, the function of Se and vitamin E are closely aligned.

The primary activities associated with bovine neutrophil function (i.e., migration, phagocytosis, and kill) are affected differently by vitamin E and/or Se status. Direct or indirection measures of migration of bovine neutrophils were enhanced when the cells were incubated in media supplemented with Se and/or vitamin E (Ndiweni and Finch, 1996,; 1995; Eicher *et al*., 1994a; Politis *et al*., 2003). Conversely, Maddox *et al*. (1999) reported that bovine neutrophils (from Se-adequate

cows) had greater adhesion to bovine mammary endothelial cells that were Se-deficient than to cells that were grown in culture with adequate Se. Although adhesion is a necessary step in neutrophil migration, these data should not be interpreted to suggest Se-deficiency increases migration. The increased adhesion could have been caused by proinflammatory signals produced by oxidative damage to the Se-deficient cells. The antioxidant capacity of the Se-adequate endothelial cells would have been greater and those cells likely had less oxidative damage. As Maddox *et al.* (1999) discussed, Se-deficiency may cause tight adhesion between neutrophils and endothelial cells and hinder migration of the neutrophil to the infection site. Cebra *et al.* (2003) reported that whole blood concentrations of Se of cows (an index of Se status) were positively correlated with neutrophil adhesion. An important difference between the studies is that in the Maddox *et al.* study, only the endothelial cells were Se-deficient, not the neutrophils. In the Cebra *et al.* study, the Se status of the neutrophils and endothelial cells varied. In vivo supplementation, as with in vitro supplementation, has resulted in increased migration. Neutrophils, but not macrophages, isolated from periparturient dairy cows supplemented with vitamin E had enhanced measures of migration (Politis *et al.*, 1996; Politis *et al.*, 2001).

With the exception of two studies (Eicher *et al.*, 1994a; Ndiweni and Finch, 1996) in which in vitro addition of vitamin E improved neutrophil phagocytosis, Se and/or vitamin E supplementation has not affected the phagocytic ability of bovine neutrophils (Grasso *et al.*, 1990; Gyang *et al.*, 1984; Hogan *et al.*, 1990; Hogan *et al.*, 1992; Ndiweni and Finch, 1996). Neutrophil kill, however, is usually improved with vitamin E and/or Se supplementation (Table 4). Neutrophils from cows that were supplemented with vitamin E and/or Se or in vitro addition of vitamin E and/or Se usually increases the production of superoxide by the cells. Superoxide is needed to kill ingested bacteria. The mode of action for increased superoxide production is not known but it may be related to overall increased viability of neutrophils when Se and vitamin E are adequate (Grasso *et al.*, 1990). Selenium and/or vitamin E supplementation usually enhances killing ability of neutrophils, however one study reported that in vitro addition of vitamin E reduced killing. This result emphasizes the fact that an optimum supplementation rate exists. Because of its antioxidant functions, excessive concentrations of vitamin E could interfere with oxidative metabolism (Okano *et al.*, 1991) and may reduce the killing ability of neutrophils.

The vast majority of studies (9 of 10) found that the addition of Se resulted in increased lymphocyte blastogenesis, but the effects of vitamin E alone are less consistent (Table 5). The effect of Se supplementation on IgG titers is often positive (Droke and Loerch, 1989; Larson *et al.*, 1988a; Swecker *et al.*, 1989;) but more commonly, titers are not affected by supplementation (Finch and Turner, 1986; Jelinek *et al.*, 1988; Lacetera *et al.*, 1999; Nemec *et al.*, 1990; Reffett *et al.*, 1988; Stabel *et al.*, 1989). Vitamin E supplementation usually does not affect IgG titers (Daniels *et al.*, 2000; Hatfield *et al.*, 2002; Nemec *et al.*, 1990; Rivera *et al.*, 2002). A consistent result with both Se and vitamin E supplementation is increased IgM titers (Nemec *et al.*, 1994; Reffett *et al.*, 1988; Stabel *et al.*, 1989; Stabel *et al.*, 1992).

The effects Se and vitamin E have on neutrophil and lymphocyte function are likely mediated via their antioxidant activities. Fatty acid hydroperoxides affect the activity of different lipoxygenases in the arachidonic acid pathways, and Se and vitamin E, via antioxidant activity, influence the concentrations of those compounds in cells. Alteration in activity of enzymes in the arachidonic

acid pathway influence the production of different prostaglandins and leukotrienes (LT). Bovine and caprine neutrophils or lymphocytes produce more LTB4 (a product of 5-lipoxygenase pathway) and less 12-HETE (a product of 12-lipoxygenase pathway) when the cells are isolated from Se-supplemented animals than Se-deficient animals (Aziz and Klesius, 1986; Cao *et al*., 1992; Maddox *et al*., 1991). Leukotriene B4 stimulated chemotaxis of neutrophils and 12-HETE can inhibit lymphocyte blastogenesis.

The preponderance of data show that Se and/or vitamin E supplementation improves immune function in ruminants. Likewise, effects of Se and/or vitamin E supplementation or status on animal health, especially mammary gland health of dairy animals, have usually been positive. Smith *et al*. (1984, 1985) was the first to show that supplementation of vitamin E and Se reduced the prevalence, duration, and severity of clinical mastitis in dairy cows. Oral supplementation of vitamin E reduced prevalence of clinical mastitis by 37% (no effect of Se injection), Se injection or oral vitamin E reduced the duration of infections by 45%, and the combination of Se and vitamin E reduced duration about 62%. Parental supplementation of ewes with vitamin E and Se reduced somatic cell count (SCC) in milk (Morgante *et al*., 1999), and oral supplementation

Table 4. Effects of vitamin E and/or Se on superoxide production (increased production is related with increased killing) and killing ability of bovine neutrophils.

Response	Reference
Superoxide production	
Production correlated positively with blood Se concentration in lactating dairy cows	Cebra *et al*., 2003
Increased with in vitro addition of vitamin E and/or Se	Ndiweni and Finch, 1996
Increased with vitamin E supplementation of periparturient dairy cows	Politis *et al*., 1995
Increased with Se supplementation of cattle	Arthur *et al*., 1981
Increased with in vitro addition of vitamin E	Higuchi and Nagahata, 2000
No effect of Se supplementation of steers	Gutzwiller, 1998
No effect (most time points) of vitamin E supplementation of dry and lactating dairy cows	Hidiroglou *et al*., 1997
Killing ability	
Increased with vitamin E (*E. coli* and *S. aureus*) or Se (*S. aureus*) supplementation of periparturient dairy cows	Hogan *et al*., 1990
Increased with Se supplementation of dairy cows	Grasso *et al*., 1990
Increased with Se/vitamin E injection to dairy cows	Gyang *et al*., 1984
Increased with vitamin E supplementation of dairy calves	Eicher *et al*., 1994b
Increased with vitamin E injection to periparturient dairy cows	Hogan *et al*., 1992
No effect with oral vitamin E to periparturient dairy cows	Hogan *et al*., 1992
Decreased with in vitro addition of vitamin E	Eicher *et al*., 1994a

of Se reduced the rate of new intramammary gland infections in dairy cows (Malbe *et al*., 1995). Prepartum supplementation of 4000 IU of vitamin E/d to dairy cows reduced new intramammary gland infections and incidence of clinical mastitis in early lactation by 63% and 89%, respectively compared to cows fed 100 IU/d (Weiss *et al*., 1997). Cows in that experiment were purposely fed diets with low concentrations of Se, and the response may have been influenced by Se status of the cows. Conversely Batra *et al*. (1992) reported that supplemental vitamin E (1000 IU/d) had no effect on prevalence of mastitis in dairy cows, but cows in that study had extremely low plasma Se concentrations (much lower than those reported by Weiss *et al*. (1997)). Following an intramammary challenge with *E. coli*, cows fed supplemental Se had lower bacterial counts in mammary secretion, and reduced severity and duration of clinical signs than cows fed no supplemental Se (Erskine *et al*., 1989). In a similar experiment with a *S. aureus,* bacterial counts in milk were lower in cows fed supplemental Se but no differences were observed in duration and severity of clinical signs (Erskine *et al*., 1990). In field-type experiments (herd level comparisons), improved Se status as measured by blood Se concentrations or GSHpx activity was associated with reduced milk SCC or mastitis (Braun *et al*., 1991; Erskine *et al*., 1987; Ndiweni *et al*., 1991; Weiss *et al*., 1990). Grace *et al*. (1997) found no relationship between concentrations of blood Se and SCC in dairy cows. Cow, not herd, was the experiment unit in that study and the cows grazed pasture. Because fresh pasture is an excellent source of vitamin E, vitamin E status of cows in that study was likely very good.

Vitamin A and B-carotene

Vitamin A has numerous functions in mammals and clearly influences immune response. Alteration of vitamin A status affects cytokine production that then influences immune cell development and function (Stephensen, 2001). The exact mode of action is not known but may

Table 5. Papers examining the effect of Se or vitamin E on bovine or ovine lymphocyte proliferation or blastogenesis.

Papers reporting positive results to Se supplementation	
Lacetera *et al*., 1999	Finch and Turner, 1989
Pollock *et al*., 1994	Larson *et al*., 1988b
Cao *et al*., 1992	Jelinek *et al*., 1988
Maddox *et al*., 1991	Turner *et al*., 1985
Stabel *et al*., 1990	
Papers reporting no effect to Se supplementation	
Sordillo *et al*., 1993	
Papers reporting positive results to Se and vitamin E supplementation	
Finch and Turner, 1989	
Papers reporting positive results to vitamin E supplementation	
Pollock *et al*., 1994	Finch and Turner, 1989
Reddy *et al*., 1986	
Papers reporting no effect to vitamin E supplementation	
Larson *et al*., 1988b	Reddy *et al*., 1986

be related to the role of vitamin A in regulating gene transcription. Dietary B-carotene, as a precursor to vitamin A, results in similar responses as dietary vitamin A. In addition to its vitamin A actions, B-carotene can react with singlet oxygen and is an important lipid soluble antioxidant in cells (Chew, 1993). Vitamin A has little, if any, cellular antioxidant activity.

In vitro supplementation of bovine neutrophils with vitamin A (retinol or retinoic acid) often reduces their phagocytic and/or killing ability (Daniel *et al*., 1991a; Eicher *et al*., 1994a; Tjoelker *et al*., 1988a). Phagocytic activity of neutrophils isolated from cows that were supplemented with vitamin A (ca. 150,000 IU/d) was the same as that of neutrophils from cows fed control diets (ca. 50,000 IU/d), and killing ability was either not affected (Tjoelker *et al*., 1990) or enhanced by vitamin A (Michal *et al*., 1994). In vivo or in vitro supplementation of B-carotene has increased killing ability of bovine neutrophils in the majority of studies (Daniel *et al*., 1991a; Michal *et al*., 1994; Tjoelker *et al*., 1988b), but Tjoelker *et al*. (1990) reported no effect of in vivo supplemental B-carotene on neutrophil function. Similar rates of B-carotene supplementation were used in the two in vivo studies (Michal *et al*., 1994; Tjoelker *et al*., 1990) but plasma B-carotene concentrations in control cows in the Tjoelker *et al*. study were higher than those in Michal *et al*. suggesting that basal status of the cows could have caused the observed differences between the two experiments. The most likely mode of action of B-carotene on neutrophil function is via its antioxidant functions, however B-carotene concentrations in neutrophils from young calves (BW = 110 kg) fed supplemental B-carotene (Chew *et al*., 1993) or from periparturient dairy cows (Weiss *et al*., 1994) is extremely low.

The effects vitamin A and/or B-carotene have on lymphocyte blastogenesis are unclear with positive, negative, and neutral responses reported (Daniel *et al*., 1991b; Franklin *et al*., 1995; Michal *et al*., 1994; Tjoelker *et al*., 1988a; 1990). Supplementation rate (especially with respect to in vitro additions), type of vitamin A (e.g., retinol vs. retinoic acid), baseline status of the animals, and stage of gestation/lactation when cells were isolated may have affected response. Lambs that were clinically deficient in vitamin A, had significantly lower titers of antigen specific IgG than lambs fed adequate vitamin A (Bruns and Webb, 1990). In other studies, in which the control animals were not clinically deficient, supplemental vitamin A did not affect antibody titers (Hidiroglou and Markham, 1996; Ritacco *et al*., 1986).

In a clinical trial with dairy cows, prepartum supplementation of vitamin A or vitamin A and B-carotene did not affect mammary gland infection rate, prevalence of clinical mastitis or SCC (Oldham *et al*., 1991). Bindas *et al*. (1984) reported that SCC was not affected when midlactation dairy cows were supplemented with B-carotene. An epidemiological study, however, reported that increased serum retinol concentrations were statistically associated with a reduced risk for clinical mastitis (LeBlanc *et al*., 2004), and Chew *et al*. (1982) reported that low concentrations of plasma vitamin A and B-carotene were associated with increased severity of mastitis in dairy cows. The prevalence of retained fetal membranes and metritis were reduced when dairy cows were supplemented with vitamin A or B-carotene (Michal *et al*., 1994). Neutrophil activity prior to calving is lower in cows destined to have retained fetal membranes than in healthy cows (Kimura *et al*., 2002) suggesting the response to supplemental vitamin A and B-carotene with respect to retained fetal membranes may be modulated via effects on the immune system.

Zinc

Zinc is a cofactor in numerous enzymes including proteases, RNA and DNA polymerase, and SOD. Zinc also is involved with regulation of gene expression at the transcription level. In nonruminants, suboptimal Zn status negatively affects immune function and disease resistance (Ibs and Rink, 2003; Walker and Black, 2004). The role of Zn as an antioxidant (i.e., SOD) and the importance of Zn to cell replication and proliferation are the two most direct connections between Zn and immune function.

Chemotaxic and phagocytic activities of human and murine neutrophils are reduced with Zn deficiency (Ibs and Rink, 2003) but no data are available on the effects of Zn status on neutrophil function in adult ruminants. Neutrophils from 12 wk old dairy calves fed diets with approximately 60, 150, or 300 mg/kg of Zn had similar phagocytic and kill activity (Kincaid *et al*., 1997), but the lowest concentration of Zn fed in that experiment probably met the Zn requirement of the calves. In nonruminants, Zn deficiency reduces proliferation of T-cells but does not greatly influence B-cells (Ibs and Rink, 2003). Cell-mediated response and lymphocyte blastogenesis after stimulation with T-cell mitogen (PHA) was reduced in Zn-deficient ruminants compared with animals adequate in Zn (Droke and Spears, 1993; Engle *et al*., 1997). The Zn deficient animals in those studies had reduced growth rates or manifested other clinical signs of Zn deficiency. In other studies that used control animals (cattle and sheep) that did not display clinical signs of Zn deficiency, additional dietary Zn has not affected lymphocyte blastogenesis, delayed hypersensitivity, or measures of humoral immunity (Droke *et al*., 1993; Droke *et al*., 1998; Droke and Spears, 1993; Kincaid *et al*., 1997; Spears and Kegley, 2002; White *et al*., 1994).

Data from controlled experiments that compared clinical health responses to supplemental Zn in ruminants are extremely limited. Feedlot steers fed a diet with 90 mg/kg of Zn (supplemental Zn from Zn-methionine) maintained lower rectal temperatures following an experimental challenge with IBR than did steers fed a diet with approximately 35 mg/kg of Zn; however, no difference in rectal temperature following challenge was observed between steers fed approximately 100 and 160 mg/kg of Zn (Chirase *et al*., 1991). Whether the effect of treatment on rectal temperatures was a response unique to Zn-methionine or a response to Zn in general could not be determined in this experiment. In another experiment using the same challenge model, steers fed a diet with supplemental Zn and Mn provided by Zn- and Mn-methionine (total diet was 75 mg/kg of Zn and 55 mg/kg Mn) had lower rectal temperatures following challenge than steers fed a diet with the same concentrations of Zn and Mn provided by oxides (Chirase and Greene, 2001). In that experiment, animals received the different minerals sources starting before birth (i.e., dams were supplemented) through arrival in the feedlot. In a summary of twelve experiments, the addition of Zn-methionine to the diet of lactating dairy cows significantly reduced average SCC, however in some of the experiments, dietary Zn concentration was confounded with the addition of Zn-methionine (Tomlinson *et al*., 2002). In four of the twelve experiments, the control diet did not meet NRC (2001) requirements for Zn. Whitaker *et al*. (1997) reported no difference in new intramammary gland infections or in prevalence of clinical mastitis between dairy cows supplemented with Zn-proteinate or inorganic Zn (source not described). Both diets contained 130 mg/kg of supplemental Zn and the basal diet contained approximately 37 mg/kg Zn. The general lack of response of measures of immune function to Zn and the positive clinical responses

to Zn (albeit based on extremely limited data) suggests that Zn may affect disease resistance via a mechanism other than neutrophil or lymphocyte function.

Other minerals and vitamins

The only known nutritional function of Co is as a component of vitamin B-12. Therefore responses to Co supplementation or deficiency are most likely caused by changes in B-12 status. Neutrophils isolated from calves deficient in Co had reduced killing ability against *Candida albicans* (MacPherson *et al*., 1987; Paterson and MacPherson, 1990). Following an experimental challenge with *Ostertagia ostertagi*, Co deficient calves had increased fecal shedding of eggs compared with Co supplemented cattle (MacPherson *et al*., 1987). Fecal shedding of eggs following a natural nematode infection was higher in Co-deficient sheep (Vellema *et al*., 1996).

Neutrophils from dairy cows given an i.v. injection of riboflavin (vitamin B_2) had increased oxidative metabolism compared to neutrophils from control cows (Sato *et al*., 1999). The injection also reduced milk SCC in cows with *S. aureus* mastitis but cure rate was not affected by riboflavin. Feedlot steers given injections of a mixture of B-vitamins and vitamin C tended to have higher IgG titers to bovine herpesvirus-1 than control steers but no effect of treatment was observed on lymphocyte blastogenesis (Dubeski *et al*., 1996). In vitro addition of vitamin D (1,25-dihydroxyvitamin D3) has various positive effects on bovine macrophage and lymphocyte functions (Nonnecke *et al*., 2003; Waters *et al*., 2001). Vitamin C is not considered essential for adult ruminants, however, some measures of neutrophil function were enhanced when steers that were given injections of dexamethasone were subcutaneously injected with ascorbic acid (Roth and Kaeberle, 1985). Ascorbic acid is probably the most important water soluble antioxidant in mammalian cells and stimulated neutrophils have extremely high concentrations of ascorbic acid (Washko *et al*., 1990). Tyler and Cummins (2003) reported that oral supplementation of vitamin C to stressed heifers reduced lymphocyte blastogenesis and reduced antigen-specific IgG titers. Intravenous administration of ascorbic acid did not have substantial effects on severity of clinical signs by dairy cows that received an intramammary challenge with lipopolysaccharide (Chaiyotwittayakun *et al*., 2002), whereas Weiss *et al*. (2004) reported a negative correlation between ascorbic acid status of dairy cows and severity of clinical signs of mastitis following an intramammary challenge with *E. coli*.

Conclusions

Many vitamins and trace minerals are unequivocally needed for immune cells to function, but demonstrating consistent effects of altered status of those nutrients on immune function is difficult with ruminants. Status of chromium, copper, selenium, zinc, B-carotene, and vitamins A and E have been shown, at least in some situations, to influence different aspects of immunity in ruminants. The lack of a measurable effect on immune function when various minerals and vitamins are supplemented does not mean the nutrients are not required for the proper functioning of the immune system of ruminants. A likely reason for the inconsistent or negligible effects observed when certain minerals and vitamins are supplemented is that the basal diet provided adequate amounts of those nutrients to maintain immune function. The relatively

consistent beneficial effects of Se and/or vitamin E supplementation may occur because control diets are sufficiently low in those nutrients.

References

Arthington, J. D., L.R. Corah & F. Blecha (1996a). The effect of molybdeun-induced copper deficiency on acute-phase protein concentrations, superoxide dismutase activity, leukocyte numbers, and lymphocyte proliferation in beef heifers inoculated with bovine herpesvirus-1. Journal of Animal Science 74, 211-217.

Arthington, J.D., L.R. Corah, F. Blecha & D.A. Hill (1995). Effect of copper depletion and repletion on lymphocyte blastogenesis and neutrophil bactericidal function in beef heifers. Journal of Animal Science 73, 2079-2085.

Arthington, J.D., L.R. Corah, J.E. Minton, T.H. Elsasser & F. Blecha (1997). Supplemental dietary chromium does not influence ACTH, cortisol, or immune response in young calves inoculated with bovine Herpesvirus-1. Journal of Animal Science 75, 217-223.

Arthington, J.D., A.R. Spell, L.R. Corah & F. Blecha (1996b). Effect of molybdenum-induced copper deficiency on in vivo and in vitro measures of neutrophil chemotaxis both before and following an inflammatory stressor. Journal of Animal Science 74, 2759-2764.

Arthur, J.R., R. Boyne, H.A. Hill & M.J. Okolow-Zubkowska (1981). The production of oxygen-derived radicals by neutrophils from selenium-deficient cattle. FEBS Letters 135, 187-190.

Aziz, E. & P.H. Klesius (1986). Effect of selenium deficiency on caprine polymorphonuclear leukocyte production of leukotriene B4 and its neutrophil chemotactic activity. American Journal of Veterinary Research 47, 426-428.

Batra, T.R., M. Hidiroglou & M.W. Smith (1992). Effect of vitamin E on incidence of mastitis in dairy cattle. Canadian Journal of Animal Science 72, 287-297.

Behne, D. & A. Kyriakopoulos (2001). Mammalian selenium-containing proteins. Annual Review of Nutrition 21, 453-473.

Bindas, E.M., F.C. Gwazdauskas, J.H. Aiello, M.L. McGilliard & C.E. Polan (1984). Reproductive and metabolic characteristics of dairy cattle supplemented with B-carotene. Journal of Dairy Science 67, 1249-1258.

Borgs, P. & B.A. Mallard (1998). Immune-endocrine interactions in agricultural species: chromium and its effect on health and performance. Domestic Animal Endocrinology 15, 431-438.

Boyne, R. & J.R. Arthur (1981). Effects of selenium and copper deficiency on neutrophil function in cattle. Journal Comparative Pathology 91, 271-276.

Boyne, R. & J.R. Arthur (1986). Effects of molybdenum or iron induced copper deficiency on the viability and function of neutrophils from cattle. Research in Veterinary Science 41, 417-419.

Braun, U., R. Forrer, W. Furer & H. Lutz (1991). Selenium and vitamin E in blood sera of cows from farms with increased incidence of disease. Veterinary Record 128, 543-547.

Broadley, C. & R.L. Hoover (1989). Ceruloplasmin reduces the adhesion and scavenges superoxide during the interaction of activated polymorphonuclear leukocytes with endothelial cells. American Journal of Pathology 135, 647-655.

Bruns, N.J. & K.E. Webb, Jr. (1990). Vitamin A deficiency: serum cortisol and humoral immunity in lambs. Journal of Animal Science 68, 454-459.

Burton, G. W. (1994). Vitamin E: molecular and biological function. Proceeding of the Nutrition Society 53, 251-262.

Burton, J.L., M.E. Kehrli, Jr., S. Kapil & R.L. Horst (1995a). Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. Journal of Leukocyte Biology 57, 317-325.

Burton, J.L., B.A. Mallard & D.N. Mowat (1993). Effects of supplemental chromium on immune responses of periparturient and early lactation dairy cows. Journal of Animal Science 71, 1532-1539.

Burton, J.L., B.A. Mallard & D.N. Mowat (1994). Effects of supplemental chromium on antibody responses of newly weaned feedlot calves to immunization with infectious bovine rhinotracheitis and parainfluenza 3 virus. Canadian Journal of Veterinary Research 58, 148-151.

Burton, J.L., B.J. Nonnecke, P.L. Dubeski, T.H. Elsasser & B.A. Mallard (1996). Effects of supplemental chromium on production of cytokines by mitogen-stimulated bovine peripheral blood mononuclear cells. Journal of Dairy Science 79, 2237-2246.

Burton, J.L., B.J. Nonnecke, T.H. Elsasser, B.A. Mallard, W.Z. Yang & D.N. Mowat (1995b). Immunomodulatory activity of blood serum from chromium-supplemented periparturient dairy cows. Veterinary Immunology and Immunopathology 49, 29-38.

Cai, T.Q., P.G. Weston, L.A. Lund, B. Brodie, D.J. McKenna & W.C. Wagner (1994). Association between neutrophil functions and periparturient disorders in cows. American Journal of Veterinary Research 55, 934-943.

Cao, Y., J.F. Maddox, A.M. Mastro, R.W. Scholz, G. Hildenbrandt & C.C. Reddy (1992). Selenium deficiency alters the lipoxygenase pathway and mitogenic response in bovine lymphocytes. Journal of Nutrition 122, 2121-2127.

Cebra, C.K., J.R. Heidel, R.O. Crisman & B.V. Stang (2003). The relationship between endogenous cortisol, blood micronutrients, and neutrophil function in postparturient Holstein cows. Journal of Veterinary Internal Medicine 17, 902-907.

Cerone, S.I., A.S. Sansinanea, S.A. Streitenberger, M.C. Garcia & N.J. Auza (1998). The effect of copper deficiency on the peripheral blood cells of cattle. Veterinary Research Communications 22, 47-57.

Cerone, S.I., A.S. Sansinanea, S.A. Streitenberger, S. Garcia & N.J. Auza (2000a). Bovine monocyte-derived macrophage function in induced copper deficiency. General Physiology and Biophysic 19, 49-58.

Cerone, S.I., A.S. Sansinanea, S.A. Streitenberger, S. Garcia & N.J. Auza (2000b). Cytochrome C oxidase, Cu,Zn-superoxide dismutase, and ceruloplasmin activities in copper-deficienct bovines. Biological Trace Element Research 73, 269-278.

Chaiyotwittayakun, A., R.J. Erskine, P.C. Bartlett, T.H. Herdt, P.M. Sears & R.J. Harmon (2002). The effect of ascorbic acid and L-histidine therapy on acute mammary inflammation in dairy cattle. Journal of Dairy Science 85, 60-67.

Chang, X., B.A. Mallard & D.N. Mowat (1994). Proliferation of peripheral blood lymphocytes of feeder calves in response to chromium. Nutrition Research 14, 851-864.

Chang, X., B.A. Mallard & D.N. Mowat (1996a). Effects of chromium on health status, blood neutrophil phagocytosis, and in vitro lymphocyte blastogensis of dairy cows. Veterinary Immunology and Immunopathology 52, 37-52.

Chang, X., B.A. Mallard, D.N. Mowat & G.F. Gallo (1996b). Effect of supplemental chromium on antibody responses of newly arrived feeder calves to vaccines and ovalbumin. Canadian Journal of Veterinary Research 60, 140-144.

Chang, X. & D.N. Mowat (1992a). Supplemental chromium for stressed and growing feeder calves. Journal of Animal Science 70, 559-565.

Chang, X. & D.N. Mowat (1992b). Supplemental chromium for stressed and growing feeder calves. Journal of Animal Science 70, 559-565.

Chang, X., D.N. Mowat & B.A. Mallard (1995). Supplemental chromium and niacin for stressed feeder calves. Canadian Journal of Animal Science 75, 351-358.

Chew, B.P. (1993). Role of carotenoids in the immune response. Journal of Dairy Science 76, 2804-2811.

Chew, B.P., L.L. Hollen, J.K. Hillers & M.L. Herlugson (1982). Relationship between vitamin A and B-carotene in blood plasma and milk and mastitis in Holsteins. Journal of Dairy Science 65, 2111-2118.

Chew, B.P., T.S. Wong & J.J. Michal (1993). Uptake of orally administered B-carotene by blood plasma, leukocytes, and lipoproteins in calves. Journal of Animal Science 71, 730-739.

Chirase, N. K. & L. W. Greene (2001). Dietary zinc and manganese sources administered from the fetal stage onwards affect immune response of transit stressed and virus infected offspring steer calves. Animal Feed Science and Technology 93, 217-228.

Chirase, N.K., D.P. Hutcheson & G.B. Thompson (1991). Feed intake, rectal temperature, and serum mineral concentrations of feedlot cattle fed zinc oxide or zinc methionine and challenged with infectious bovine rhinotracheitis virus. Journal of Animal Science 69, 4137-4145.

Daniel, L.R., B.P. Chew, T.S. Tanaka & L.W. Tjoelker (1991a). B-carotene and vitamin A effects on bovine phagocyte function in vitro during the peripartum period. Journal of Dairy Science 74, 124-131.

Daniel, L.R., B.P. Chew, T.S. Tanaka & L.W. Tjoelker (1991b). In vitro effects on B-carotene and vitamin A on peripartum bovine peripheral blood monomuclear cell proliferation. Journal of Dairy Science 74, 911-915.

Daniels, J.T., P.G. Hatfield, D.E. Burgess, R.W. Kott & J.G.P. Bowman (2000). Evaluation of ewe and lamb immune response when ewes were supplemented with vitamin E. Journal of Animal Science 78, 2731-2736.

Droke, E.A., G.P. Gengelbach & J.W. Spears (1998). Influence of level and source (inorganic or organic) or zinc supplementation on immune function in growing lambs. Asian Australas Journal of Animal Science 11, 139-144.

Droke, E.A. & S.C. Loerch (1989). Effects of parenteral selenium and vitamin E on performance, health, and humoral immune response of steers new to the feedlot environment. Journal of Animal Science 67, 1350-1359.

Droke, E.A. & J.W. Spears (1993). In vitro and in vivo immunological measurements in growing lambs fed diets deficient, marginal, or adequate in zinc. Journal of Nutritional Immunology 2, 71-90.

Droke, E.A., J.W. Spears, T.T. Brown & M.A. Qureshi (1993). Influence of dietary zinc and dexamethasone on immune responses and resistance to *Pasteurella hemolytica* challenge in growing lambs. Nutrition Research 13, 1213-1226.

Dubeski, P.L., J.M. d'Offay, F.N. Owens & D.R. Gill (1996). Effects of B vitamin injection on bovine herpesvirus-1 infection and immunity in feed-restricted beef calves. Journal of Animal Science 74, 1367-1374.

Eicher, S.D., J.L. Morrill & F. Blecha (1994a). Vitamin concentration and function of leukocytes from dairy calves supplemented with vitamin A, vitamin E, and B-carotene in vitro. Journal of Dairy Science 77, 560-565.

Eicher, S.D., J.L. Morrill, F. Blecha, C.G. Chitko-McKown, N.V. Anderson & J.J. Higgins (1994b). Leukocyte functions of young dairy calves fed milk replacers supplemented with vitamins A and E. Journal of Dairy Science 77, 1399-1407.

Engle, T.E., C.F. Nockels, C.V. Kimberling, D.L. Weaber & A.B. Johnson (1997). Zinc repletion with organic or inorganic forms of zinc and protein turnover in marginally zinc-deficient calves. Journal of Animal Science 75, 3074-3081.

Erskine, R.J. & P.C. Bartlett (1993). Serum concentrations of copper, iron, and zinc during *Escherichia coli*-induced mastitis. Journal of Dairy Science 76, 408-413.

Erskine, R.J., R.J. Eberhart, P.J. Grasso & R.W. Scholz (1989). Induction of *Escherichia coli* mastitis in cows fed selenium-deficient or selenium-supplemented diets. American Journal of Veterinary Research 50, 2093-2100.

Erskine, R.J., R.J. Eberhart, L.J. Hutchinson & R.W. Scholz (1987). Blood selenium concentrations and glutathionine peroxidase activities in dairy herds with high and low somatic cell counts. Journal of the American Veterinary Medical Association 190, 1417-1421.

Erskine, R.J., R.J. Eberhart & R.W. Scholz (1990). Experimentally induced *Staphylococcus aureus* mastitis in selenium-deficient and selenium-supplemented dairy cows. Journal of the American Veterinary Medicine Association 51, 1107-1111.

Faldyna, M., A. Pechova & J. Krejci (2003). Chromium supplementation enhances antibody response to vaccination with tetanus toxoid in cattle. Journal of Veterinary Medicine (Series B) 50, 326-331.

Finch, J.M. & R.J. Turner (1986). Selenium supplementation in lambs: effects on antibody responses to a salmonella vaccine. Veterinary Record. 119, 430-431.

Finch, J.M. & R.J. Turner (1989). Enhancement of ovine lymphocyte responses: a comparison of selenium and vitamin E supplementation. Veterinary Immunology and Immunopathology 23, 245-256.

Franklin, S.T., J.W. Young, R.L. Horst, T.A. Reinhardt & B.J. Nonnecke (1995). Administration of 13-cis-retinoic acid to dairy cattle. 1. Plasma retinoid concentrations and lymphocyte blastogenesis. Journal of Dairy Science 78, 62-69.

Franklin, S.T., J.W. Young & B.J. Nonnecke (1994). Proliferation and phenotype of bovine mononuclear leukocytes in cultures stimulated by pokeweed mitogen. Journal of Dairy Science 77, 3592-3600.

Gengelbach, G.P. & J.W. Spears (1998). Effects of dietary copper and molybdenum on copper status, cytokine production, and humoral immune response of calves. Journal of Dairy Science 81, 3286-3292.

Gengelbach, G.P., J.D. Ward, J.W. Spears & T.T. Brown, Jr. (1997). Effects of copper deficiency and copper deficiency coupled with high dietary iron or molybdenum on phagocytic cell function and response of calves to a respiratory disease challenge. Journal of Animal Science 75, 1112-1118.

Gentry, L.R., J.M. Fernandez, T.L. Ward, T.W. White, L.L. Southern, T.D. Bidner, J. Thompson, D.L., D.W. Horohov, A.M. Chapa & T. Sahlu (1999). Dietary protein and chromium tripicolinate in Suffolk wether lambs: Effects on production characteristics, metabolic and hormonal responses, and immune status. Journal of Animal Science 77, 1284-1294.

Goff, J.P. & J.R. Stabel (1990). Decreased plasma retinol, a-tocopherol, and zinc concentration during the periparturient period: effect of milk fever. Journal of Dairy Science 73, 3195-3199.

Grace, N.D., S.O. Knowles & J. Lee (1997). Relationship between blood Se concentrations and milk somatic cell counts in dairy cows. New Zealand Veterinary Journal 45, 171-172.

Grasso, P., R.W. Scholz, R.J. Erskine & R.J. Eberhart (1990). Phagocytosis, bactericidal activity, and oxidative metabolism of mammary neutrophils from dairy cows fed selenium-adequate and selenium-deficient diets. American Journal of Veterinary Research 51, 269-275.

Griffin, M.D., N.Z. Xing & R. Kumar (2003). Vitamin D and its analogs as regulators of immune activation and antigen presentation. Annual Review of Nutrition 23, 117-145.

Gutzwiller, A. (1998). Erythrocyte resistance to oxidative damage and leucocyte capacity to reduce nitroblue tetrazolium in selenium-deficient cattle. Zentralblatt fur Veterinarmedizin Reihe A 45, 271-278.

Gyang, E.O., J.B. Stevens, S.D. Tsitsamis & E.A. Usenik (1984). Effects of selenium-vitamin E injection on bovine polymorphonucleated leukocytes phagocytosis and killing of *Staphylococcus aureus*. American Journal of Veterinary Research 45, 175-177.

Hatfield, P.G., B.L. Robinson, D.L. Minikhiem, R.W. Kott, N.I. Roth, J.T. Daniels & C.K. Swenson (2002). Serum alpha-tocopherol and immune function in yearling ewes supplemented with zinc and vitamin E. Journal of Animal Science 80, 1329-1334.

Hellman, N.E. & J.D. Gitlin (2002). Ceruloplasmin metabolism and function. Annual Review of Nutrition 22, 439-458.

Hidiroglou, M., T.R. Batra & X. Zhao (1997). Bioavailability of vitamin E compounds and the effect of supplementation on release of superoxide and hydrogen peroxide by bovine neutrophils. Journal of Dairy Science 80, 187-193.

Hidiroglou, M. & F. Markham (1996). Effect of oral supplements of vitamin A on the plasma retinol levels in calves and their immunological unresponsiveness. Reproduction, Nutrition, Development 36, 467-472.

Higuchi, H. & H. Nagahata (2000). Effects of vitamins A and E on superoxide production and intracellular signaling of neutrophils in Holstein calves. Canadian Journal of Veterinary Research 64, 69-75.

Hill, A.W., A.L. Shears & K.G. Hibbitt (1979). The pathogenesis of experimental *Escherichia coli* mastitis in newly calved dairy cows. Research in Veterinary Science 26, 97-101.

Hogan, J.S., K.L. Smith, W.P. Weiss, D.A. Todhunter & W.L. Shockey (1990). Relationships among vitamin E, selenium, and bovine blood neutrophils. Journal of Dairy Science 73, 2372-2378.

Hogan, J.S., W.P. Weiss, D.A. Todhunter, K.L. Smith & P.S. Schoenberg (1992). Bovine neutrophil responses to parenteral vitamin E. Journal of Dairy Science 75, 399-405.

Ibs, K.H. & L. Rink (2003). Zinc-altered immune function. Journal of Nutrition 133, 1452S-1456S.

Jelinek, P.D., T. Ellis, R.H. Wroth, S.S. Sutherland, H.G. Masters & D.S. Petterson (1988). The effect of selenium supplementation on immunity, and the establishment of an experimental *Haemonchus contortus* infection in weaner merino sheep fed a low selenium diet. Australian Veterinary Journal 65, 214-217.

Jones, D.G. & N.F. Suttle (1981). Some effects of copper deficiency on leucocyte function in sheep and cattle. Research in Veterinary Science 31, 151-156.

Kegley, E.B. & J.W. Spears (1995). Immune response, glucose metabolism, and performance of stressed feeder calves fed inorganic or organic chromium. Journal of Animal Science 73, 2721-2726.

Kegley, E.B., J.W. Spears & T.T. Brown, Jr. (1997). Effect of shipping and chromium supplementation on performance, immune response, and disease resistance of steers. Journal of Animal Science 75, 1959-1964.

Kegley, E.B., J.W. Spears & J.T.T. Brown (1996). Immune response and disease resistance of calves fed chromium nicotinic acid complex or chromium chloride. Journal of Dairy Science 79, 1278-1283.

Kimura, K., J.P. Goff, M.E. Kehrli & T.A. Reinhardt (2002). Decreased neutrophil function as a cause of retained placenta in dairy cattle. Journal of Dairy Science 85, 544-550.

Kincaid, R.L., B.P. Chew & J.D. Cronrath (1997). Zinc oxide and amino acids as sources of dietary zinc for calves: effects on uptake and immunity. Journal of Dairy Science 80, 1381-1388.

Lacetera, N., U. Bernabucci, B. Ronchi & A. Nardone (1999). The effects of injectable sodium selenite on immune function and milk production in Sardinian sheep receiving adequate dietary selenium. Veterinary Research 30, 363-370.

Larson, H.J., K. Moksnes & G. Overnes (1988a). Influence of selenium on antibody production in sheep. Research in Veterinary Science 45, 4-10.

Larson, H.J., G. Overnes & K. Moksnes (1988b). Effect of selenium on sheep lymphocyte responses to mitogens. Research in Veterinary Science 45, 11-15.

LeBlanc, S.J., T.H. Herdt, W.M. Weymour, T.F. Duffield & K.E. Leslie (2004). Peripartum serum vitamin E, retinol, and beta-carotene in dairy cattle and their associations with disease. Journal of Dairy Science 87, 609-619.

Lindell, S.A., R.T. Brandt, J.E. Minton, F. Blecha, G.L. Stokka & C.T. Milton (1994). Supplemental Cr and revaccination effects on performance and health of newly weaned calves. Journal of Animal Science 77 (Suppl. 1), 133 (abstract).

Mackenzie, R.C., J.R. Arthur, S.M. Miller, T.S. Rafferty & G.J. Beckett (2002). Selenium and the immune system. In: P.C. Calder, C.J. Field & N.S. Gill (eds) Nutrition and Immune Function. CABI, Oxford, UK, p. 229-250.

MacPherson, A., D. Gray, G.B.B. Mitchell & C.N. Taylor (1987). Ostertagia infection and neutrophil function in cobalt-deficient and cobalt-supplemented cattle. British Veterinary Journal 143, 348-353.

Maddox, J.F., K.M. Aherne, C.C. Reddy & L.M. Sordillo (1999). Increased neutrophil adherence and adhesion molecule mRNA expression in endothelial cells during selenium deficiency. Journal Leukocyte Biology 65, 658-664.

Maddox, J.F., Y.Z. Cao, A.M. Mastro, R.W. Scholz, G. Hildenbrandt & C.C. Reddy (1991). Increased 12-HETE production in bovine lymphocytes during selenium deficiency. Biochemical and Biophysical Research Communications 181, 389-395.

Malbe, M., M. Klaassen, W. Fang, V. Myllys, M. Vikerpuur, K. Nyholm, W. Sankari, K. Suoranta & M. Sandholm (1995). Comparisons of selenite and selenium yeast feed supplements on Se-incorporation, mastitis, and leukocyte function in Se-deficient dairy cows. Journal of Veterinary Medicine (Series A) 42, 111-121.

Mathison, G.W. & D.F. Engstrom (1995). Chromium and protein supplements for growing-finishing beef steers fed barley-based diets. Canadian Journal of Animal Science 75, 549-558.

McMurray, D.N. (1984). Cell-mediated immunity in nutritional deficiency. Progress in Food and Nutritional Science 8, 193-228.

Meydani, S.N. & A.A. Beharka (1998). Recent developments in vitamin E and immune response. Nutrition Reviews 56, S38-S48.

Michal, J.J., L.R. Heirman, T.S. Wong, B.P. Chew, M. Frigg & L. Volker (1994). Modulatory effects of dietary B-carotene on blood and mammary leukocyte function in periparturient dairy cows. Journal of Dairy Science 77, 1408-1421.

Moonsie-Shageer, S. & D.N. Mowat (1993). Effect of level of supplemental chromium on performance, serum constituents, and immune status of stressed feeder calves. Journal of Animal Science 71, 232-238.

Morgante, M., D. Beghelli, M. Pauselli, P. DallAra, M. Capuccella & S. Ranucci (1999). Effect of administration of vitamin E and selenium during the dry period on mammary health and milk cell counts in dairy ewes. Journal of Dairy Science 82, 623-631.

Mowat, D.N., X. Chang & W.Z. Yang (1993). Chelated chromium for stressed feeder calves. Canadian Journal of Animal Science 73, 49-55.

National Research Council (2001). Nutrient Requirements of Dairy Cattle, 7th revised edition. National Academy Press, Washington DC, 381pp.

Ndiweni, N., T.R. Field, M.R. Williams, J.M. Booth & J.M. Finch (1991). Studies on the incidence of clinical mastitis and blood levels of vitamin E and selenium in dairy herds in England. Veterinary Record 128, 86-88.

Ndiweni, N. & J.M. Finch (1995). Effects of in vitro supplementation of bovine mammary gland macrophages and peripheral blood lymphocytes with alpha-tocopherol and sodium selenite: implications for udder defences. Veterinary Immunology and Immunopathology 47, 111-121.

Ndiweni, N. & J.M. Finch (1996). Effects of in vitro supplementation with alpha-tocopherol and selenium on bovine neutrophil functions: implications for resistance to mastitis. Veterinary Immunology and Immunopathology 51, 67-78.

Nemec, M., G. Butler, M. Hidiroglou, E. R. Farnworth & K. Nielsen (1994). Effect of supplementing gilts' diets with different levels of vitamin E and different fats on the humoral and cellular immunity of gilts and their progeny. Journal of Animal Science 72, 665-676.

Nemec, M., M. Hidiroglou, K. Nielsen & J. Proulx (1990). Effect of vitamin E and selenium supplementation on some immune parameters following vaccination against brucellosis in cattle. Journal of Animal Science 68, 4303-4309.

Niederman, C.N., D. Blodgett, D.E. Eversole, G.G. Schurig & C.D. Thatcher (1994). Effect of copper and iron on neutrophil function and humoral immunity of gestating beef cows. Journal of the American Veterinary Medical Association 204, 1796-1800.

Nockels, C.K., J. Debonis & J. Torrent (1993). Stress induction affects copper and zinc balance in calves fed organic and inorganic copper and zinc sources. Journal of Animal Science 71, 2539-2545.

Nonnecke, B.J., J.L. Burton & M.E. Kehrli, Jr. (1997). Associations between function and composition of blood mononuclear leukocyte populations from Holstein bulls treated with dexamethasone. Journal of Dairy Science 80, 2403-2410.

Nonnecke, B.J., W.R. Waters, M.R. Foote, R.L. Horst, M.A. Fowler & B.L. Miller (2003). In vitro effects of 1,25-dihydroxyvitamin D3 on interferon-gamma and tumor necrosis factor-alpha, secretion by blood leukocytes from young and adult cattle vaccinated with *Mycobacterium bovis*. International Journal of Vitamin and Nutrition Research 73, 235-244.

Okano, T., H. Tamai & M. Mino (1991). Superoxide generation in leukocytes and vitamin E. International Journal of Vitamin and Nutrition 61, 20-26.

Oldham, E.R., R.J. Eberhart & L.D. Muller (1991). Effects of supplemental vitamin A and B-carotene during the dry period and early lactation on udder health. Journal of Dairy Science 74, 3775-3781.

Olkowski, A.A., S.R. Gooneraatne & D.A. Christensen (1990). Effects of high sulphur content and varied concentrations of copper, molybdenum and thiamine on in vitro phagocytic and candidacidal activity of neutrophils in sheep. Research in Veterinary Science 48, 82-86.

Paterson, J.E. & A. MacPherson (1990). The influence of low cobalt intake on the neutrophil function and severity of Ostertagia infection in cattle. British Veterinary Journal 146, 519-530.

Percival, S.S. (1998). Copper and immunity. American Journal of Clinical Nutrition 67 (Suppl.), 1064S-1068S.

Politis, I., M. Hidiroglou, T.R. Batra, J.A. Gilmore, R.C. Gorewit & H. Scherf (1995). Effects of vitamin E on immune function of dairy cows. American Journal of Veterinary Research 56, 179-184.

Politis, I., N. Hidiroglou, J.H. White, J.A. Gilmore, S.N. Williams, H. Scherf & M. Frigg (1996). Effects of vitamin E on mammary and blood leukocyte function, with emphasis on chemotaxis, in periparturient dairy cows. American Journal of Veterinary Research 57, 468-471.

Politis, I., A. Voudouri, I. Bizelis & G. Zervas (2003). The effect of various vitamin E derivatives on the urokinase- plasminogen activator system of ovine macrophages and neutrophils. British Journal of Nutrition 89, 259-265.

Politis, L., N. Hidiroglou, F. Cheli & A. Baldi (2001). Effects of vitamin E on urokinase-plasminogen activator receptor expression by bovine neutrophils. American Journal of Veterinary Research 62, 1934-1938.

Pollock, J.M., J. McNair, S. Kennedy, D.G. Kennedy, D.M. Walsh, E.A. Goodall & A.D. Crockard (1994). Effects of dietary vitamin E and selenium on in vitro cellular immune responses in cattle. Research in Veterinary Science 56, 100-107.

Prohaska, J.R. & M.L. Failla (1993). Copper and immunity. In: D.M. Klurfeld (ed) Human Nutrition-A Comprehensive Treatise. Plenum Press, New York, pp. 309-332.

Reddy, P.G., J.L. Morrill, H.C. Minocha, M.B. Morrill, A.D. Dayton & R.A. Frey (1986). Effect of supplemental vitamin E on the immune system of calves. Journal of Dairy Science 69, 164-171.

Reffett, J.K., J.W. Spears & T.T. Brown, Jr. (1988). Effect of dietary selenium and vitamin E on the primary and secondary immune response in lambs challenged with parainfluenza 3 virus. Journal of Animal Science 66, 1520-1528.

Ritacco, K.A., C.F. Nockels & R.P. Ellis (1986). The influence of supplemental vitamins A and E on ovine humoral immune response. Proceedings of the Society for Experimental Biology and Medicine 182, 393-398.

Rivera, J.D., G.C. Duff, M.L. Galyean, D.A. Walker & G.A. Nunnery (2002). Effects of supplemental vitamin E on performance, health, and humoral immune response of beef cattle. Journal of Animal Science 80, 933-941.

Roth, J.A. & M.L. Kaeberle (1985). In vivo effect of ascorbic acid on neutrophil function in healthy and dexamethasone-treated cattle. American Journal of Veterinary Research 46, 2434-2436.

Sato, S., H. Hori & K. Okada (1999). Effect of vitamin B-2 on somatic cell counts in milk of clinical Staphylococcus aureus mastitis. Journal of Veterinary Medical Science 61, 569-571.

Scaletti, R.W., D.S. Trammell, B.A. Smith & R.J. Harmon (2003). Role of dietary copper in enhancing resistance to Escherichia coli mastitis. Journal of Dairy Science 86, 1240-1249.

Shankar, A.H. & A.S. Prasad (1998). Zinc and immune function: the biological basis of altered resistance to infection. American Journal of Clinical Nutrition 68 (Suppl.), 447S-463S.

Smith, K.L., H.R. Conrad, B.A. Amiet & D.A. Todhunter (1985). Incidence of environmental mastitis as influenced by vitamin E and selenium. Kieler Milchwirtshaftliche Forsch 37, 482-486.

Smith, K.L., J.H. Harrison, D.D. Hancock, D.A. Todhunter & H.R. Conrad (1984). Effect of vitamin E and selenium supplementation on incidence of clinical mastitis and duration of clinical symptoms. Journal of Dairy Science 67, 1293-1300.

Sordillo, L.M., C.R. Hicks, R. Wilson & J. Maddox (1993). Effects of selenium status on bovine mononuclear cell function. Journal of Veterinary Medicine (Series A) 40, 615-623.

Spears, J.W. (1989). Zinc methionine for ruminants: relative bioavailability of zinc in lambs and effects of growth and performance of growing heifers. Journal of Animal Science 67, 835-843.

Spears, J.W. (2000). Micronutrients and immune function in cattle. Proceedings of the Nutrition Society 59, 587-594.

Spears, J.W. (2003). Trace mineral bioavailability in ruminants. Journal of Nutrition 133, 1506S-1509S.

Spears, J.W. & E.B. Kegley (2002). Effect of zinc source (zinc oxide vs zinc proteinate) and level on performance, carcase characteristics, and immune response of growing and finishing steers. Journal of Animal Science 80, 2747-2752.

Stabel, J.R., B.J. Nonnecke & T.A. Reinhardt (1990). Effect of in vitro selenium repletion on bovine lymphocyte proliferation. Nutrition Reserach 10, 1053-1059.

Stabel, J.R., T.A. Reinhardt, M.A. Stevens, J.M.E. Kehrli & B.J. Nonnecke (1992). Vitamin E effects on in vitro immunoglobulin M and interleukin-1B production and transcript in dairy cattle. Journal of Dairy Science 75, 2190-2198.

Stabel, J.R., J.W. Spears & J.T.T. Brown (1993). Effect of copper deficiency on tissue, blood characteristics, and immune function of calves challenged with infectious bovine rhinotracheitis virus and pasteurella hemolytica. Journal of Animal Science 71, 1247-1255.

Stabel, J.R., J.W. Spears, J.T.T. Brown & J. Brake (1989). Selenium effects on glutathione peroxidase and the immune response of stressed cavles challenged with *Pasteurella hemolytica*. Journal of Animal Science 67, 557-564.

Stephensen, C.B. (2001). Vitamin A, infection, and immune function. Annual Review of Nutrition 21, 167-193.

Suttle, N.F. & D.G. Jones (1989). Recent developments in trace element metabolism and function: Trace elements, disease resistance and immune responsiveness in ruminants. Journal of Nutrition 119, 1055-1061.

Swecker, W.S., D.E. Eversole, C.D. Thatcher, D. Blodgett, G.G. Schurig & J.B. Meldrum (1989). Inlfuence of supplemental selenium on humoral immune responses in weaned beef calves. American Journal of Veterinary Research 50, 1760-1763.

Tjoelker, L.W., B.P. Chew, T.S. Tanaka & L.R. Daniel (1988a). Bovine vitamin A and B-carotene intake and lactation status. 2. Responsiveness of mitogen-stimulated peripheral blood lymphocytes to vitamin A and B-carotene challenge in vitro. Journal of Dairy Science 71, 3120-3128.

Tjoelker, L.W., B.P. Chew, T.S. Tanaka & L.R. Daniel (1988b). Bovine vitamin A and B-carotene intake and lactational status. 1. Responsiveness of peripheral blood polymorphonuclear leukocytes to vitamin A and B-carotene challenge in vitro. Journal of Dairy Science 71, 3112-3120.

Tjoelker, L.W., B.P. Chew, T.S. Tanaka & L.R. Daniel (1990). Effect of dietary vitamin A and B-carotene on polymorphonuclear leukocyte and lymphocyte function in dairy cows during the early dry period. Journal of Dairy Science 73, 1017-1022.

Tomlinson, D.J., M.T. Socha, C.J. Rapp & A.B. Johnson (2002). Summary of twelve trials evaluating the effect of feeding complexed zinc methionine on lactation performance of dairy cattle. Journal of Dairy Science 85 (Suppl. 1), 107 (abstract).

Torre, P.M., R.J. Harmon, R.W. Hemken, T.W. Clark, D.S. Trammell & B.A. Smith (1996). Mild copper insufficiency depresses blood neutrophil function in dattle cattle. Journal of Nutritional Immunology 4, 3-24.

Turner, R. J., L. E. Wheatley & N. F. Beck (1985). Stimulatory effects of selenium on mitogen responses in lambs. Veterinary Immunology and Immunopathology 8, 119-124.

Tyler, P J. & K A. Cummins (2003). Effect of dietary ascorbyl-2-phosphate on immune function after transport to a feeding facility. Journal of Dairy Science 86, 622-629.

Vellema, P., V.P.M.G. Rutten, A. Hook, L. Moll & G.H. Wentink (1996). The effect of cobalt supplementation on the immune response in vitamin B12 deficient Texel lambs. Veterinary Immunology and Immunopathology 55, 151-161.

Walker, C F. & R.E. Black (2004). Zinc and the risk for infectious disease. Annual Review of Nutrition 24, 255-275.

Ward, J.D., G.P. Gengelbach & J.W. Spears (1997). The effects of copper deficiency with or without high dietary iron on molybdenum on immune function of cattle. Journal of Animal Science 75, 1400-1408.

Ward, J.D. & J.W. Spears (1999). The effects of low-copper diets with or without supplemental molybdenum on specific immune responses of stressed cattle. Journal of Animal Science 77, 230-237.

Ward, J.D., J.W. Spears & E.B. Kegley (1993). Effect of copper level and source (copper lysine vs copper sulfate) on copper status, performance, and immune response in growing steers fed diets with or without supplemental molybdenum and sulfur. Journal of Animal Science 71, 2748-2755.

Washko, P., D. Rotrosen & M. Levine (1990). Ascorbic acid accumulation in plated human neutrophils. FEBS Letters 260, 101-104.

Waters, W. R., B. J. Nonnecke, T. E. Rahner, M. V. Palmer, D. L. Whipple & R. L. Horst (2001). Modulation of mycobacterium bovis-specific responses of bovine peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D3. Clinical and Diagnostic Laboratory Immunology 8, 1204-1212.

Weiss, W.P., J.S. Hogan & K.L. Smith (2004). Changes in vitamin C concentrations in plasma and milk from dairy cows after an intramammary infusion of *Escherichia coli*. Journal of Dairy Science 87, 32-37.

Weiss, W.P., J.S. Hogan, K.L. Smith & K.H. Hoblet (1990). Relationships among selenium, vitamin E, and mammary gland health in commercial dairy herds. Journal of Dairy Science 73, 381-390.

Weiss, W.P., J.S. Hogan, K.L. Smith & S.N. Williams (1994). Effect of dietary fat and vitamin E on a-tocopherol and B-carotene in blood of peripartum cows. Journal of Dairy Science 77, 1422-1429.

Weiss, W.P., J.S. Hogan, D.A. Todhunter & K.L. Smith (1997). Effect of vitamin E supplementation in diets with a low concentration of selenium on mammary gland health of dairy cows. Journal of Dairy Science 80, 1728-1737.

Whitaker, D.A., H.F. Eayres, K. Aitchison & J.M. Kelly (1997). No effect of a dietary zinc proteinate on clinical mastitis, infection rate, recovery rate, and somatic cell count in dairy cows. Veterinary Journal 153, 197-204.

White, C.L., H.J. Kyme & M.J. Barnes (1994). Marginal zinc deficiency in pregnant Merino ewes. Proceedings of the Nutritional Society of Australia 18, 85.

Woolliams, C., N.F. Suttle, J.A. Wooliams, D.G. Jones & G. Wiener (1986). Studies on lambs genetically selected for low and high copper status. 1. Differences in mortality. Animal Production 43, 293-301.

Wright, A.J., D.N. Mowat & B.A. Mallard (1994). Supplemental chromium and bovine respiratory disease vaccines for stressed feeder calves. Canadian Journal of Animal Science 74, 287-295.

Wright, C.L., J.W. Spears, T.T. Brown, K.E. Lloyd & M.E. Tiffany (2000). Effects of chromium and copper on performance and immune function of stressed steers. Journal of Animal Science 78 (Suppl. 2), 1 (abstract).

Yang, W.Z., D.N. Mowat, A. Subiyatno & R.M. Liptrap (1996). Effects of chromium supplementation on early lactation performance of Holstein cows. Canadian Journal of Animal Science 76, 221-230.

Xin, Z., D.F. Waterman, R.W. Hemken & R.J. Harmon (1991). Effects of copper status on neutrophil function, superoxide dismutase, and copper distribution in steers. Journal of Dairy Science 74, 3078-3085.

Part VII: Nutrition and stress physiology

Feeding management and stress in calves

A.M. de Passillé and J. Rushen
Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, PO Box 1000, 6947 Highway 7, Agassiz, BC, Canada

Abstract

Deprivation of sucking behaviour, low milk intakes and individual housing are some of the factors thought to reduce calf welfare in calf rearing systems. To assess the extent of problems associated with sucking behaviour and to find alternative housing and feeding systems, it is necessary to understand the basic causes of behaviour. Milk-fed calves reared separately from their mothers often show much non-nutritive sucking, which takes the form of cross-sucking when the calves are reared in groups. Non-nutritive sucking appears to be a part of normal nursing and is often associated with teat switching and butting, which reflect a low rate of milk transfer during the nursing. Performance of sucking behaviour by calves, independent of milk intake affects secretion of CCK, insulin and oxytocin and may lead to greater relaxation of the calves. This behaviour appears to be stimulated by the taste of milk, especially the taste of lactose, and is reduced when the calves can suck a teat either during or after a milk meal. Increasing the opportunity for calves to suck, either by reducing milk flow rate during milk feeding or by providing a dry teat after a meal, can substantially reduce cross-sucking and improve group-housing. Increasing the quantity of milk fed to calves improves growth and feed conversion and leads to greater efficiency of automated milk feeders.

Keywords: animal welfare, stress, housing, sucking behaviour, calves

Introduction

The most commonly used methods of rearing and managing milk-fed calves for both dairy and veal production have been criticized for the effect they have on the calves' welfare. Our research program has focused upon identifying the main sources of stress for milk-fed calves and in developing cost-effective alternatives that can improve calf health and welfare while maintaining good productivity. We have adopted the approach of developing these alternatives through a better understanding of the basic causes of the calves' behaviour.

Among the various sources of stress that have been suggested as constituting threats to the welfare of milk-fed calves, we will focus upon three: the deprivation of sucking behaviour, low milk intake, and the use of individual housing. This article summarizes the results of our research into these potential sources of stress.

Deprivation of sucking behaviour

In "nature" (and in most beef production systems) unweaned calves obtain their milk from suckling at their mother. For dairy and veal calves, however, calves drink their milk either by sucking on a teat feeder or by drinking directly from a bucket. While the former obviously differs

in many respects from normal nursing, it does provide the calves with the opportunity to perform sucking behaviour. In contrast, when milk is provided in a bucket, the calves do not need to suck during milk ingestion. How much of a threat to their welfare does this constitute? A number of studies over the years have compared the growth and health of calves that were fed milk from a bucket or from a teat and, generally, have found little difference. Most recently, for example, Veissier *et al.* (2002) found identical weight gains in bucket and teat-fed calves, and, although there was a trend for health problems to be more evident in bucket-fed calves, these differences were not statistically significant. In ad lib fed calves, however, growth rates of teat-fed calves can be higher than bucket-fed calves due to a higher intake of milk (Hammell *et al.*, 1988).

However, there are a number of behavioural and more subtle physiological consequences of not allowing calves to suck their milk. For example, Veissier *et al.* (2002) noted that teat fed calves lay down sooner and spent more time lying down in a fully relaxed position, for example, with their heads resting on their flanks or on the ground. It is in these relaxed postures that sleep occurs (Hänninen *et al.*, 2002). De Passillé *et al.* (1993) noted also that concentrations of CCK and insulin in the portal vein were higher after a meal when the calves were able to suck a dry teat. More recently, other physiological consequences of the method of consuming milk have been found. Lupoli *et al.* (2001) have found that calves that have to suck to obtain milk show larger increases in plasma oxytocin during milk ingestion that do bucket-fed calves. Veissier *et al.* (2002) found some cardiovascular effects associated with sucking. While the full consequences of these differences for calf welfare have yet to be elucidated, these results show that deprivation of sucking behaviour cannot be assumed to have no consequences for the calf even if growth and overall health is not affected.

The most obvious consequences of not allowing milk fed-calves to suck a teat, however, is the large amount of non-nutritive sucking that occurs. In individually-housed calves, this often takes the form of sucking at parts of the pen or the milk bucket. When milk-fed calves are housed in groups, the most common form of non-nutritive sucking is cross-sucking (recently reviewed by Jensen, 2003), where one calf will suck principally at the belly, inguinal area or the mouth and ears of another calf (e.g. Lidfors, 1993; Loberg and Lidfors, 2001; Margerison *et al.*, 2003). This behaviour can lead to injuries and urine drinking and may lead to "milk stealing" from lactating cows (Keil *et al.*, 2000; Keil and Langhans, 2001; Lidfors and Isberg 2003). However, there is little evidence that cross-sucking is associated with poorer health of calves (Lidfors 1993). The occurrence of this behaviour discourages dairy producers from keeping milk-fed calves in groups, despite the labour advantages of this form of rearing (Kung *et al.*, 1997; de Passillé *et al.*, 2004).

The causes and consequences of sucking behaviour

To be able to fully control cross-sucking and to decide on the importance of sucking deprivation for animal welfare, we need to understand better the causes and consequences of sucking behaviour. Most obviously, the motivation to suck would seem to result from the calves' hunger, and, under normal circumstances, the performance of sucking, leading to the ingestion of milk, would reduce this hunger. This simple negative-feedback model of motivation is illustrated in Figure 1. However, there are many reasons for thinking this model is at best oversimplified and at worst wrong.

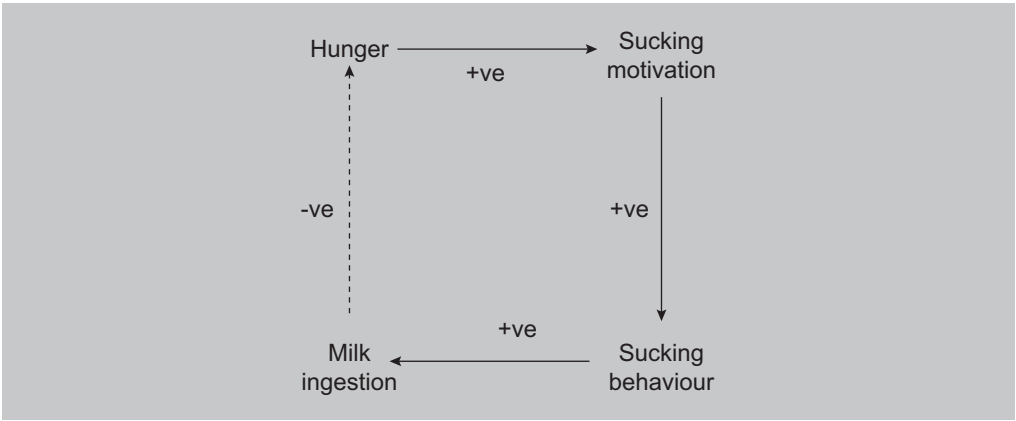

Figure 1. A simple negative feedback model of sucking motivation. The model assumes that sucking is motivated primarily by the calf's hunger. Under normal circumstances, sucking results in milk ingestion, which reduces the hunger and so reduces sucking motivation. The results of much research now show that this model is over-simplistic.

For example, if sucking motivation was increased by hunger and consequently reduced by the ingestion of milk, we would expect to find sucking motivation to be highest (and sucking behaviour to be most evident) before the meal and to be lower after the meal. However, several studies have shown that this does not appear to be the case. De Passillé and Caza (1997) found that cross-sucking in group-housed, milk-fed veal calves was highest immediately after the meal (Figure 2). Loberg and Lidfors (2001) also report cross-sucking occurring after a milk meal. Cross-sucking was reduced when the calves could suck a dry-teat after the meal (Figure 2) even if this did not alter milk ingestion (de Passillé and Caza, 1997). Finally, cross-sucking is greatly reduced (and can almost disappear) when the calves are weaned off milk (Lidfors, 1993). Clearly, the simple negative feed-back model presented in Figure 1 does not explain these results, and we need better understand sucking motivation.

Is non-nutritive sucking "abnormal"?

We are often tempted to call behaviours, such as non-nutritive sucking, "abnormal" when the function of those behaviours is not obvious to us. However, studies of a number of species show that some non-nutritive sucking is a regular part of normal nursing and this appears to be the case also for cattle. Lidfors *et al.* (1994) examined in detail the behaviours that occurred during a normal nursing in cattle and described three apparent phases. The first phase consisted of short periods of sucking with much butting and switching between teats. The second phase consisted of longer bouts of uninterrupted sucking and the third phase was similar to the first, with frequent teat switching and butting. Lidfors *et al.* (1994) suggested that milk ingestion occurred primarily during the second phase and that the first and third phases were essentially non-nutritive sucking with little milk ingestion. Experimental support for this was provided by Mayntz and Costa (1998) who observed the changes in the calves' sucking behaviour during a nursing when the cow was injected with oxytocin. The occurrence of milk ejection (stimulated by the oxytocin

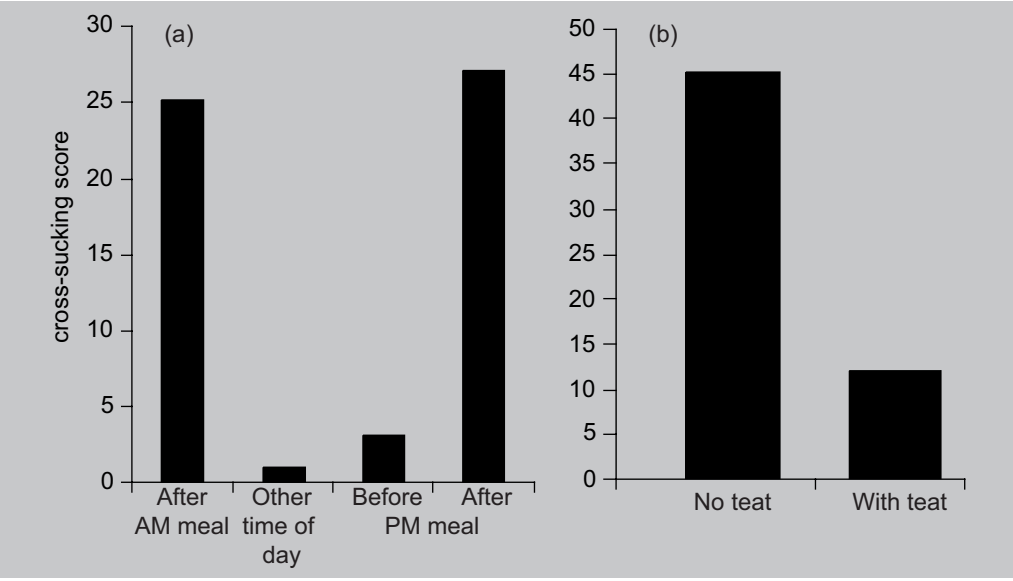

Figure 2. Occurrence of cross-sucking in group-housed milk-fed veal calves (a) at different times of the day (after the morning milk meal, at another time of the day not associated with meals, and before and after the afternoon milk meal) and (b) after a milk meal when the calves were able to suck a dry teat after the meal or did not have access to the teat. The sucking score was based on the number of calves cross-sucking and the number of observation periods during which they cross-sucked. Based on the results of de Passillé and Caza (1997).

injection) was apparent in long bouts of uninterrupted sucking. This was preceded and succeeded by periods of shorter bouts of sucking. Together, these studies strongly suggest that some non-nutritive sucking does occur during normal nursing in cattle, and that the longest periods of non-nutritive sucking occur after milk ingestion. As de Passillé and Caza's (1997) results showed, this is closely related to the timing of cross-sucking and is consistent with the fact that milk ingestion stimulates sucking. This may have also functional consequences during nursing: continued sucking after milk ejection would ensure complete emptying of the udder improving growth and survival of the calf.

An experimental model for studying the causes and consequences of cross-sucking

We have developed an experimental model (de Passillé *et al*., 1992) which allows us to closely control the factors that might influence sucking behaviour in order to understand sucking motivation. Milk-fed calves are either provided milk in a bucket or milk is injected directly into their mouths and they are then allowed to suck a dry-teat. This model allows us to separate out the effects of milk ingestion from the effects of performing sucking behaviour alone. The first question we asked was whether sucking motivation was reduced by the ingestion of milk. We allowed the calves to drink four litres, two litres or no milk from a bucket. Surprisingly, we found that the duration of sucking behaviour afterwards was lowest (not highest) when the calves did

not drink milk and that the actual amount of milk had no effect (Rushen and de Passillé, 1995). Clearly, the ingestion of milk appeared to be stimulating rather than reducing sucking behaviour, which explains why cross-sucking is high after milk meals.

To further understand this effect, we altered the composition of the liquid that was given to the calves (de Passillé *et al*., 1997). We compared water, a suspension of grain in water, and milk replacer at various concentrations. We found that milk replacer stimulated more sucking that either water or the grain suspension, and that an increased concentration of milk replacer had a larger effect (Figure 3).

We then used reconstituted milk to examine the relative importance of the main components of milk: lactose, casein, the whey proteins and fat. By reducing and increasing the concentrations of each of these independently, we showed that the concentration of lactose appeared to be the factor that had the largest influence upon the amount of non-nutritive sucking that occurred.

We then examined whether the performance of sucking behaviour without the ingestion of milk reduced sucking motivation. To do this, we injected into the mouths of the calves a small quantity of milk (10mls). Half the calves were then allowed to suck a dry teat while the others were not allowed to suck. Following this, we injected a second small portion of milk and allowed all the calves to suck the dry teat. The calves that had been able to suck after the first portion of milk showed significantly less sucking after the second portion (Rushen and de Passillé, 1995). Together, these results suggested that it is the performance of sucking behaviour *per se*, rather than the ingestion of milk, which reduces sucking motivation.

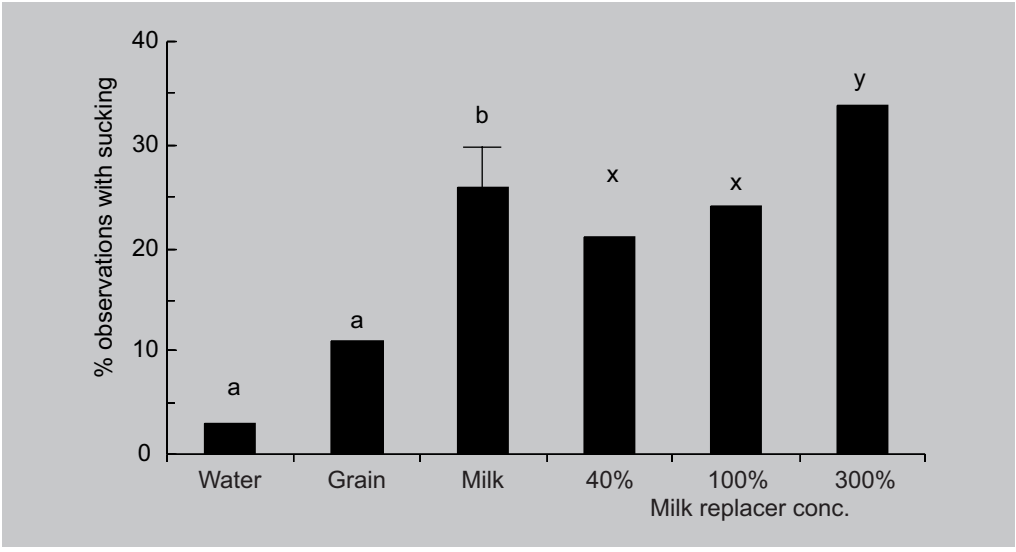

Figure 3. Occurrence of non-nutritive sucking immediately after calves ingested various liquids. (Drawn from data presented in de Passillé et al.*, 1997). Different superscripts (a,b and x,y) indicate significant differences.*

Haley *et al*. (1998a) supported this interpretation by examining the effects of reducing the duration of milk flow during sucking. Calves were fed with a simple teat-feeding system and washers with holes of various diameters were used to alter the rate of milk flow. The smaller washers reduced the rate that the calves ingested milk and consequently increased the duration of the nutritive sucking. Once the milk had been ingested, the duration of the non-nutritive sucking that occurred was lower with the smaller diameter washers. That slower milk flow rates can reduce cross-sucking between calves has been confirmed by Loberg and Lidfors (2001).

The conclusion of these experiments is that sucking motivation is increased rather than reduced by the ingestion of milk, at least during the immediate post-prandial period. The taste of the milk, specifically the taste of the lactose, is the main stimulatory factor. The sucking motivation is reduced by the performance of sucking behaviour rather than by milk ingestion. This is illustrated in Figure 4.

Suckling is more than sucking

During suckling, it is the performance of sucking itself which is responsible for milk ingestion, but many other behaviours occur during a bout of nursing. As Lidfors *et al*. (1994) described, nursing by cattle involves much switching between teats and butting by the calf at the udder. We developed a milk-feeding system for calves that allowed us to examine some of the factors controlling the occurrence of these behaviours. Calves were trained to suck milk from a teat that was connected to a bucket. As we described above, Haley *et al*. (1998b) examined the effect of the flow rate of milk during sucking by altering the size of the orifice in the tube through which the milk flowed. When milk flow was slow, the calves were seen to butt more frequently at the teat. A high frequency of butting could be produced simply by repeatedly turning off the milk supply. To examine the factors that lead the calves to switch between teats, de Passillé *et al*. (1996) adapted

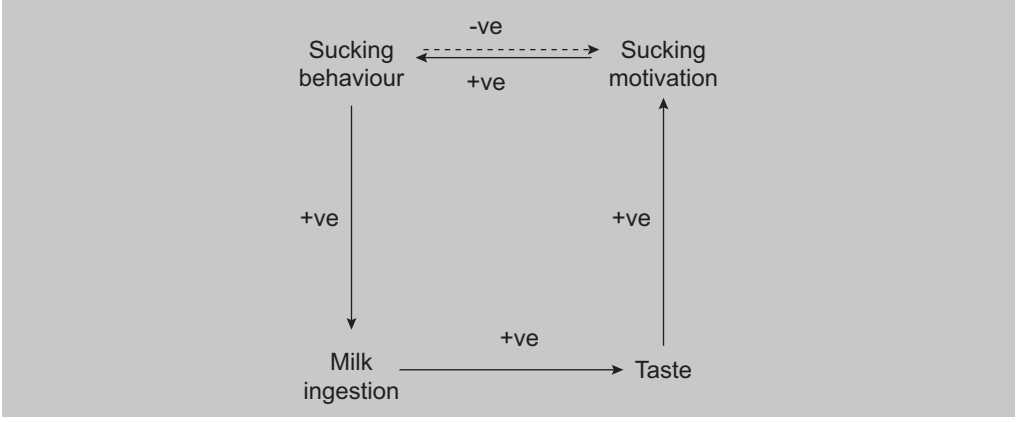

Figure 4. The figure summarizes the research showing that sucking motivation is reduced by the performance of sucking behaviour itself rather than by milk ingestion. Indeed, ingestion of milk stimulates rather than reduces sucking motivation, primarily through the taste of lactose, which is an example of the positive feedback commonly found in motivational systems.

this feeding system so that the calves could chose between two teats and found that the milk flow rate had a large effect on teat switching. Generally, the calves spent more time sucking on the teat with the highest flow rate and would often switch teats when the flow rate of their favoured teat was reduced. Most teat switching occurred when both teats had low flow rates. In this artificial feeding model, the extent that the calves butted and switched between teats reflected the rate at which the milk was flowing. Is this also true during normal nursing?

Altering the calf's hunger and milk availability during nursing

We answered this question by allowing calves to continue nursing from their mothers twice a day for eight weeks. The cows were also milked twice a day and the nursing occurred two hours after the afternoon milking. By weighing the calf before and after nursing, we were able to measure the amount of milk drunk by the calf. We varied the amount of milk available to the calf by changing how much milk we removed from the cow during the milking that preceded the nursing. We took detailed recordings of the calves' behaviour during the nursing. Reducing milk availability resulted in a reduction in the amount of milk consumed and in large increases in the duration of sucking and the frequency of switching between teats and butting (Figure 5). The effects of teat switching and butting are very similar to the effects found in our experimental teat-feeding model when milk flow rate is reduced. Together the results show that a high frequency of butting and teat switching is an indication of low milk availability and supports Lidfors *et al.*'s (1994) and Mayntz and Costa's (1998) suggestion that non-nutritive suckling is occurring when teat switching and butting are most frequent.

We also varied the calf's hunger either by tube feeding the calf with milk before the nursing or by skipping one milk meal. Increasing the calves' hunger resulted in similar changes as reducing milk availability except that the effects were much smaller and the amount of milk consumed was increased (Figure 5).

The fact that similar changes in suckling behaviour were found when the amount of milk actually consumed was both increased (due to increased calf hunger) and reduced (due to reduced milk availability) shows why it is so difficult to estimate the amount of milk consumed during a nursing from measures of the calves' sucking behaviour (Cameron, 1998).

Milk quantity

In many cases, milk-fed dairy calves are fed only limited amounts of milk, which is substantially less than what they would normally drink during a nursing or where milk is available ad lib (e.g. Hammon *et al.*, 2002; Hepola, 2003). There is increasing evidence that this is insufficient to reduce their hunger and is not optimal for growth and development. For example, in North America it is common for milk-fed calves to receive only 4-6L of milk a day. We found that when calves were allowed to nurse the cow twice a day, their daily intake was between 9 and 10L and their growth rates over the 8 week milk feeding period were substantially higher (~1 kg /d) than those of calves fed the usual 4L (~0.5 kg/d). Recent research has shown similar effects for calves fed ad lib milk from a teat feeding system (Appleby *et al.*, 2001; Jasper and Weary, 2002). Ad lib fed calves consumed between 6 and 12 L of milk a day and growth rates were substantially higher

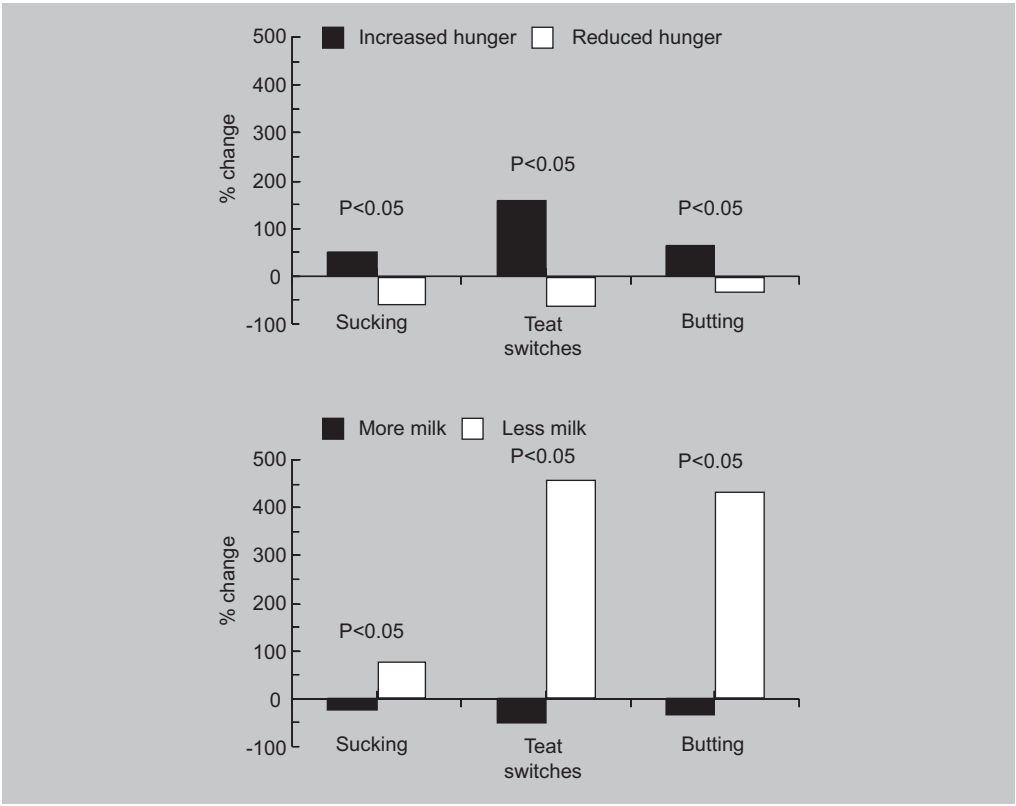

Figure 5. Changes in duration of sucking, and frequency of teat switches and butting when a calf is suckling from a cow either when the calf's hunger is increased or reduced (upper figure) or when milk availability in the udder is increased or reduced (lower figure). (Data from de Passillé et al.*, 1996).*

over the milk feeding period. When weaned off milk, the ad-lib fed calves did show a slight check in growth over the following week (similarly reported by Hepola, 2003), but normal growth soon recommenced and they maintained their weight advantage over the conventionally fed calves. The higher growth rates of ad lib fed calves has been shown also to occur during the first week of life (Hammon *et al.*, 2002), a period when bucket-fed calves can show no obvious weight gains and where health risks are highest. Furthermore, the feed conversion efficiency for ad lib feeding can be substantially higher than when calves are fed restricted amounts, approaching feed conversion efficiencies found for other species such as piglets and lambs (Diaz *et al.*, 2001). However, other studies have reported lower feed conversion efficiencies (Hammon *et al.*, 2002; Hepola, 2003).

We do not yet know what advantages occur to the calves' welfare as a result of this higher milk intake. However, it is most likely that the calves' level of hunger is lower. Calves receiving smaller quantities of milk a day make more unrewarded visits (i.e. when no milk was available) to the feeder, strongly suggesting that the smaller daily portions were not satisfying the calves (Hammon

et al., 2002; Jensen and Holm, 2003). Furthermore, the amount of cross-sucking that occurs is reduced when milk portions are increased from 2.5L to 5L (Jung and Lidfors, 2001). Although there seem to be several advantages with increased milk intakes, we still have to find the optimal management techniques for supplying this (Hepola, 2003).

Individual versus group housing

One of the more controversial aspects of calf rearing (especially for veal production) is the use of individual housing. In Europe, concern about the welfare of individually housed animals led the governments of the EU to ban individual housing of calves after the age of 8 weeks, except for purposes of veterinary treatment. Since most dairy calves are weaned off milk by 8 weeks, this law does not have as much importance for dairy production as for veal production. In North America, where there are no such restrictions, individual housing remains the predominant way of housing unweaned dairy and veal calves.

However, there is growing interest in the use of group housing of milk-fed calves partly because of the economic advantages associated with the lower labour requirements, especially when automated milk-feeding equipment is used (Kung *et al*., 1997). Recently, in a comparison of milk-fed calves that were housed individually, de Passillé *et al*. (2004) showed that the daily labour requirements to care for individually housed calves was an average total of 4 hours / calf compared to only 1.4 hours / calf when a group housing system was used with automated milk-feeding equipment.

Furthermore, group housing has several potential advantages for calf welfare in that it generally allows calves to have more space for exercise, which is important for the welfare of growing animals, and does allow some social contact to occur (Chua *et al*., 2002). However, there are also some risks to animal welfare associated with group housing. Two of the main concerns that producers have with group-housing for unweaned calves are the possibility that cross-sucking can occur, and the higher incidence of illness and mortality.

It seems likely that the problem of increased disease incidence can be dealt with by good management. For example, two large scale epidemiological studies have shown that calf mortality and morbidity can be as low in group-housing as in individual housing providing that the groups are kept small (~7-10 animals) (Lösinger and Heinrichs, 1997; Svensson *et al*., 2003). We found no differences in growth or health of calves kept in groups of 6-12 animals compared to individually housed animals (de Passillé *et al*., 2004) and Chua *et al*. (2002) reported no differences in health status between pair-housed and individually housed calves. Indeed, Kung *et al*. (1997) reported fewer health problems in group-housed calves fed with an automated feeding system compared to individually housed calves. Furthermore, the use of automated milk-feeding equipment has the potential to collect data on feed intakes of individual calves and changes in feed intake can be used to help identify individual calves that become ill, since drops in food intake are traditionally used as indicators of illness. Small groups seem to be the key to successful management of group-housing: large groups have been reported several times to be associated with increased aggression and competition over feeders (Jensen, 2003).

Although some studies do report problems of cross-sucking in group-housed calves (e.g. Keil and Langhans, 2001; Lidfors and Isberg, 2003), others report only a low incidence of the behaviour (e.g. Mattiello *et al*., 2002). The problem of cross-sucking can also be controlled by appropriate feeding management. For example, use of automated milk feeders or other teat feeding systems allow the calves to suck their milk through a teat, which reduces the incidence of cross-sucking (e.g. Loberg and Lidfors, 2001; Lidfors and Isberg, 2003). Even providing a dry teat after the meal (de Passillé and Caza, 1997) can substantially reduce cross-sucking. However, use of teat feeding systems are not invariably associated with reduced cross-sucking (e.g. Veissier *et al*., 2002), suggesting that the details of feeding management are important. Slower rates of milk delivery can reduce the incidence of cross-sucking (Loberg and Lidfors 2001). Automated milk feeding systems can be designed to allow the calves to suck the teat for a sufficient time after the meal to satisfy their sucking motivation. For example, cross-sucking is increased if teat-fed calves are not able to continue to suck the teat after milk ingestion (Jung and Lidfors, 2001) but a swing-door (described in Jensen, 2003) can prevent calves from being displaced from the teat by other calves, which allows them to perform longer bouts of non-nutritive sucking on the teat and which results in a lower incidence of cross-sucking that is associated with milk ingestion (Weber and Wechsler, 2001). We found few instances of cross-sucking (1 instance / 10 calves /24 h) when calves in groups of 6-12 were fed with such a system (de Passillé *et al*., 2004). Displacements of calves from the teat feeder can also be reduced by feeding the calves a larger quantity of milk (Jensen and Holm, 2003). With appropriate management, we believe that group-housing has considerable potential to improve both the economics of calf rearing and the welfare of milk-fed calves.

Conclusions

The research shows that, in order to solve the issue of cross-sucking in group housed calves, it is necessary to understand the causes and consequences of sucking behaviour. Furthermore, it is likely that we still do not know what is the correct amount to feed young calves. Together the evidence is accumulating that, at least in some parts of the world, we are feeding insufficient quantities to satisfy the calves. By controlling cross-sucking and by feeding the appropriate amounts of milk, we will be able to improve the performance of group housing for milk-fed calves, which will allow us to take advantage of the potential this form of housing has for improved calf welfare and productivity. Feeding larger quantities of milk will help us better take advantage of the calf's full potential for growth.

Acknowledgements

The research discussed in this article was funded by the Natural Sciences and Engineering Research Council of Canada, Agriculture and Agri-Food Canada and De Laval Inc. We thank Marjolaine St. Louis, Isabelle Blanchet and Keith Carter for their help with this research, and Dan Weary, Lene Munksgaard, Margit Bak Jensen, Lene Lidfors and Isabelle Veissier for very helpful discussions.

References

Appleby, M.C., D.M. Weary & B. Chua (2001). Performance and feeding behaviour of calves on ad libitum milk from artificial teats. Applied Animal Behaviour Science, 74, 191-201.

Cameron, E.Z. (1998). Is sucking behaviour a useful predictor of milk intake. Animal Behaviour, 56, 521-532.

Chua, B., Coenen, E., J. van Delen & D.M. Weary (2002). Effects of pair versus individual housing on the behavior and performance of dairy calves. Journal of Dairy Science, 85, 360-364.

de Passillé, A.M.B. & N. Caza (1997). Cross-sucking by calves occurs after meals and is reduced when calves suck a dry teat. Journal of Dairy Science, 80 (Suppl 1), 229.

de Passillé, A.M.B., R. Christopherson & J. Rushen (1993). Nonnutritive sucking by the calf and postprandial secretion of insulin, CCK and gastrin. Physiology and Behavior, 54, 1069-1073.

de Passillé, A.M.B., J.H.M. Metz, P. Mekking & P.R. Wiepkema (1992). Does drinking milk stimulate sucking in young calves? Applied Animal Behaviour Science, 1992, 34, 23-36.

de Passillé, A.M.B., G. Richer, J. Morrissette & J. Rushen (1996). Quantity of milk in the udder, the level of hunger, and sucking behaviour of the nursing calf. In I.J.H. Duncan, T. M. Widowski and D. B. Haley (Eds.). Proceedings of the 30th International Congress of the International Society for Applied Ethology, Guelph, Ontario. p58.

de Passillé, A.M.B., J. Rushen & M. Janzen (1997). Some aspects of milk that elicit non-nutritive sucking in the calf. Applied Animal Behaviour Science, 53, 167-173.

de Passillé, A.M., J. Rushen & D. Weary (2004). Designing good environments and management for calves. Advances in Dairy Technology, 16, 75-89.

Diaz, M.C., M. Van Amburgh, J.M. Smith, J.M. Kelsey & E.L. Hutten (2001). Composition of growth of Holstein calves fed milk replacer from birth to 105-kilogram body weight. Journal of Dairy Science, 84, 830-842.

Haley, D., J. Rushen, I. Duncan, T. Widowski & A.M.B. de Passillé (1998a). Effects of resistance to milk flow and provision of hay on non-nutritive sucking by dairy calves. Journal of Dairy Science, 81, 2165-2172.

Haley, D., J. Rushen, I. Duncan, T. Widowski & A.M.B. de Passillé (1998b). Butting by calves (*Bos taurus*) and rate of milk flow. Animal Behaviour, 56, 275-285.

Hammell, K.L., J.H.M. Metz & P. Mekking (1988). Sucking behaviour of dairy calves fed milk ad libitum by bucket or teat. Applied Animal Behaviour Science, 20, 275-285.

Hammon, H.M., G. Schiessler, A. Nussbaum & J.W. Blum (2002). Feed intake patterns, growth performance, and metabolic and endocrine traits in calves fed unlimited amounts of colostrum and milk by automate, starting in the neonatal period. Journal of Dairy Science, 85, 3352-3362.

Hänninen, L., H. Hepola, J. Rushen, A.M. De Passillé, P. Pursiainen, V.M. Tuure, L. Syrjälä-Qvist, M. Pyykkönen & H. Saloniemi (2002). Resting behaviour, growth and diarrhoea incidence rate of young dairy calves housed individually or in groups in warm or cold buildings. Acta Agricultural Scandinavica Section A Animal-Science, 53, 21-28.

Hepola, H. (2003). Milk feeding systems for dairy calves in groups, effects on feed intake, growth and health. Applied Animal Behaviour Science, 80, 233-243.

Jasper, J. & D.M. Weary (2002). Effects of ad libitum milk intake on dairy calves. Journal of Dairy Science, 85, 3054-3058.

Jensen, M.B. (2003). The effects of feeding method, milk allowance and social factors on milk feeding behaviour and cross-sucking in group housed dairy calves. Applied Animal Behaviour Science, 80, 191-206.

Jensen, M.B. & L. Holm (2003). The effect of milk flow rate and milk allowance on feeding related behaviour in dairy calves fed by computer controlled milk feeders. Applied Animal Behaviour Science, 82, 87-100.

Jung, J. & L. Lidfors (2001). Effects of amount of milk, milk flow and access to a rubber teat on cross-sucking and non-nutritive sucking in dairy calves. Applied Animal Behaviour Science, 72, 201-213.

Keil, N.M. & W. Langhans (2001). The development of intersucking in dairy calves around weaning. Applied Animal Behaviour Science, 72, 295-308.

Keil, N.M., L. Audigé & W. Langhans (2000). Factors associated with intersucking in Swiss dairy heifers. Preventive Veterinary Medicine, 45, 305-323.

Kung, L., S. Demarco, L.N. Siebenson, E. Joyner, G.F.W. Haenlein & R.M. Morris (1997). An evaluation of two management systems for rearing calves fed milk replacer. Journal of Dairy Science, 80, 2529-2533.

Lidfors, L. (1993). Cross-sucking in group-housed dairy calves before and after weaning off milk. Applied Animal Behaviour Science, 38, 15-24.

Lidfors, L. & L. Isberg (2003). Intersucking in dairy cattle—review and questionnaire. Applied Animal Behaviour Science, 80, 207-231.

Lidfors, L., P. Jensen & B. Algers (1994). Suckling in free-ranging beef cattle, temporal patterns of suckling bouts and effects of age and sex. Ethology, 98, 321-332.

Loberg, J. & L. Lidfors (2001). Effect of milk flow rate and presence of a floating nipple on abnormal sucking between dairy calves. Applied Animal Behaviour Science, 72, 189-199.

Lösinger, W.C. & A.J. Heinrichs (1997). Management practices associated with high mortality among preweaned dairy heifers. Journal of Dairy Research. 64, 1-11.

Lupoli, B., B. Johansson, K. Uvnas-Moberg & K. Svennersten-Sjaunja (2001). Effect of suckling on the release of oxytocin, prolactin, cortisol, gastrin, cholecystokinin, somatostatin and insulin in dairy cows and their calves. Journal of Dairy Research, 68, 175-187.

Margerison, J.K., T.R. Preston, N. Berry & C.J.C. Phillips (2003). Cross-sucking and other oral behaviours in calves, and their relation to cow suckling and food provision. Applied Animal Behaviour Science, 80, 277-286.

Mattiello, S., E. Canali, V. Ferrante, M. Caniatti, F. Gottardo, G. Cozzi, I. Andrighetto & M. Verga (2002). The provision of solid feeds to veal calves, II. Behavior, physiology, and abomasal damage. Journal of Animal Science, 80, 367-75.

Mayntz, M. & R. Costa (1998). Effect of pharmacologically induced changes in milk ejection on suckling in *Bos Taurus*. Physiology and Behavior, 65, 151-156.

Rushen, J. & A.M. de Passillé (1995). The motivation of non-nutritive sucking in calves, *Bos taurus*. Animal Behaviour, 49, 1503-1510.

Svensson, C., K. Lundborg, U. Emanuelson & S-O. Olsson (2003). Morbidity in Swedish dairy calves from birth to 90 days of age and individual calf-level risk factors for infectious diseases. Preventive Veterinary Medicine, 58, 179-197.

Veissier, I., A.M. de Passillé, G. Després, J. Rushen, I. Charpentier, A.R. Ramirez de la Fe & P. Pradel (2002). Does nutritive and non-nutritive sucking reduce other oral behaviors and stimulate rest in calves. Journal of Animal Science, 80, 2574-2587.

Weber, R. & B. Wechsler (2001). Reduction in cross-sucking in calves by the use of a modified automatic teat feeder. Applied Animal Behaviour Science, 72, 215-223.

Effects of nutrition on stress reactivity

L. Munksgaard, M.S. Herskin, P. Løvendahl and J.B. Andersen
Danish Institute of Agricultural Sciences, Research Centre Foulum, P.O. Box 50, DK-8830 Tjele, Denmark

Abstract

Evidence suggests that diet composition and energy intake can affect responses to stress in non-ruminant mammals, possibly mediated through effects on brain serotonin levels and/or HPA-axis regulation. In ruminants, feeding and fasting can induce changes in the activity of the HPA-axis. However, in lactating cows, total energy intake does not seem to affect the plasma concentrations of cortisol, ACTH or the reactivity at the adrenal and the pituitary level. But in early lactation low energy density in the diet can induce increased cortisol response to acute stress, suggesting a change in the regulation of CRF at CNS level, which may be due to feedback from the metabolic status of the animals. Increased milk yield requires higher energy intake, thereby leading to increased time spent eating, which may results in shortage of time for other important behaviours. We found that eating time was negatively correlated with lying time in first lactation cows, and under time constraints cows did to some degree trade off eating time for lying time. We suggest that under commercial conditions some high producing cows may not be able to fulfil their behavioural needs for eating and lying.

Keywords: nutrition, HPA-axis, behaviour, stress reactivity

Introduction

Under commercial conditions dairy cows are exposed to a variety of potentially challenging events in their surroundings; e.g. moving, hoof trimming, regrouping, isolation, and aggression from conspecifics. Such events will often include novel stimuli, which can provoke acute stress responses and negative emotions such as fear (Boissy and Bouissou, 1995; Herskin, 2004). In the same way conditioned fear can provoke stress reactions i.e. conditioned fear of humans induce increased heart rate, plasma cortisol concentrations and decreased milk yield (Rushen *et al*., 1999). In so far the nutrition affects the emotional response to a given stressor; this will have an impact on the welfare of the animal. Furthermore, the level of energy mobilization to such events may affect the degree of deleterious effects. Even though much have been and can be done to limit the amount of stressors cattle are subjected to under commercial conditions, it is worth exploring whether steps can be taken to reduce the reactivity toward stress through feeding.

The physiological condition of a high producing dairy cow changes dramatically through lactation as a result of changes in milk yield, feed intake and hormonal changes due to gestation. Cows are expected to respond by adjusting their activities and behaviour throughout the day in order to fulfil their needs in the best possible way. It is well documented that composition of the feed can affect eating time (Phillips, 2002). However, with increasing milk production there is a demand for increased energy intake. Depending on the composition of the feed ration this demand for

increased energy intake requires increased eating time as well, and thus leaves the cow less time for other important activities.

The increased incidence of disorders and metabolic imbalance in dairy cows has been ascribed to the large increase in the milk production obtained during the last decades (Ingvartsen *et al*., 2003). However, metabolic imbalance might affect the stress reactivity. In rodents there is growing evidence for a close relationship between the nutritional status and both behavioural and HPA-axis responses to stress (Dallman *et al*., 2003). The HPA-axis is one of the primary adaptive mechanisms in response to stressors (e.g. review by Sapolsky, 2002). In various animal species including ruminants increased HPA-axis activity is typically seen in connection with acute stress and acts to mobilize energy to escape or defeat the threat. Furthermore, the corticotropin-releasing factor (CRF) system at the level of the central nervous system (CNS) is involved in the regulation of feed intake as well as behavioural responses to stress (Dunn and Berridge, 1990; Koob, 1999). Repeated or long-term exposure to stressors can affect the regulation of the HPA-axis in cattle, both at the adrenal level (Ladewig and Smidt, 1989, Fisher *et al*., 1997) and at the pituitary level (Munksgaard *et al*., 1999, Fisher *et al*., 2002). However, in ruminants the regulation of the HPA-axis during repeated or long-term exposure to stressors is not well understood, and there has only been limited focus on the effects of nutrition on reactivity of the HPA-axis in ruminants.

In this paper we discuss relations between 1) energy intake/diet composition and activity as well as reactivity of the HPA-axis; 2) diet composition, serotonin and behavioural stress responses; and 3) effects of diet composition and energy intake on time budgets.

Changes in HPA-axis activity induced by feeding and fasting

Ingestion of feed in dairy cows has been shown to induce a temporary decrease in plasma cortisol concentrations (Samuelsson *et al*., 1996, Johansson *et al*., 1999), and a postprandial decrease in plasma cortisol was also observed in milk feed calves (Gardy-Godillot *et al*., 1989). In ewes fed only once daily there was a significant diurnal variation in plasma cortisol concentration, with a decrease in cortisol concentration after feeding, but this was not observed in multi-fed ewes (Simonetta *et al*., 1991). In the once daily fed ewes, the decrease in cortisol concentration after feeding coincided with an increase in plasma glucose, thus Simonetta *et al.* (1991) suggested that variation in glucose concentration might affect cortisol secretion.

Fasting beef bulls and steers for 24-30 hours led to increased concentrations of cortisol (Ward *et al*., 1992), and concentrations of glucocorticoids were higher 22 to 23 hours after feeding compared to 2 to 3 hours after feeding in both pre- and post pubertal dairy heifers (Sejrsen *et al*., 1983). These results agree with Mills and Jenny (1979), who found that glucocorticoid concentration increased with time after feeding. In lactating dairy cows, fasting also led to increased plasma concentrations of cortisol as well as a tendency to increased cortisol response to milking (Samuelsson *et al*., 1996). However, Kazmer *et al.* (1985) found no differences in plasma cortisol concentrations between heifers fasted for a longer period (five days) and fed animals, in agreement with results from McCann and Hansel (1986).

Thus both feeding and fasting can induce changes in plasma cortisol levels, but feeding induced changes are less pronounced in ruminants fed ad libitum or several times a day.

Baseline cortisol levels in relation to diet composition and total energy intake

Total energy intake can be controlled both by restricting food intake of a given ration or by changes in the composition of the diet. As suggested in the previous paragraph, diurnal variation in HPA-axis activity under some circumstances relates to feeding, thus timing of blood sampling when comparing the effects of different feeding strategies can be critical. The activity in the HPA-axis in lactating animals differs from non-lactating growing animals (Beerda *et al*., 2004, Munksgaard Unpublished). Furthermore, in studies done with cows in early lactation the negative energy balance observed in most cows' regardless of variation in total energy intake might override effects of energy intake. Laugero (2001) suggest that glucocorticoid feed back in the HPA-axis depends on some aspect(s) of energy balance. Therefore it is not surprising that results concerning the effects of energy intake on baseline levels of plasma concentrations of cortisol are conflicting. Two decades ago Sejrsen *et al.* (1983) and Mills and Jenny (1979) found, that dairy heifers with a higher energy intake showed increased concentrations of glucocorticoid, whereas variation in the energy intake did not affects baseline concentrations of plasma cortisol in a number of other studies (Table 1). Furthermore in one study with steers an increase in plasma concentrations of cortisol in limited fed animals compared to animals fed a high-energy diet for ad libitum intake has been reported (Trenkle and Topel, 1978).

*Table 1. Levels of plasma concentrations of cortisol (*glucocorticoid) in cattle depending on energy intake.*

Animals	Feeding		Cortisol, high vs low	Reference
	High	Low		
Jersey, early lact. (n=32)	4% of body weight, DM basis	3% of body weight, DM basis	NS	Fisher *et al*., 2002
Holstein, early lact., (n=39)	75% concentrate	25% concentrate	NS	Munksgaard *et al*., 2004
Cross breed cows (n=10)	124% metabolizable energy requirement	82% metabolizable energy requirement	NS	Hall *et al*.,1991
Angus/Hereford, midt lact, (n=14)	26.5 Mcal ME	15.2 Mcal ME	NS	Schrick *et al*., 1990
Holstein heifers (n=11)	Ad lib (gain 1218 g/d)	Restricted (613 g/d)	↑*	Sejrsen *et al*., 1983
Holstein heifers (n=12)	30:70 Ratio (DM basis) Cornsilage: concentrate	80:20 Ratio (DM basis) Cornsilage: concentrate	↑*	Mills and Jenny, 1979
Cross breed beef steers (n=?)[1]	Ad libitum, daily gain 1.0-1.2 kg/day	Limited, daily gain 0.7-0.85	↓	Trenkle and Topel, 1978
[1]24 animals included in the experiment, but not all on treatments full/limited				

HPA-axis reactivity to acute stress is modulated by energy intake

In dairy cows dietary energy level can affect plasma concentrations of glucose and insulin (Andersen *et al*., 2004). During stress the availability of simple glucose for CNS is important, and as their name implies glucocorticoids have a central role in energy mobilization during stress (e.g. Moberg, 1985; Sapolsky, 2002). In rodents, there is evidence that the reactivity of the HPA-axis is affected by the nutritional status of the animals. Fasted rats showed higher corticosterone responses to short term restraint than fed rats (Akana *et al*., 1994), and rats with ad libitum access to sucrose exhibited lower ACTH and corticosterone responses to short term restraint than rats without access to sucrose (Strack *et al*., 1997). These results suggest that the amount of easily accessible energy may affect the need for activity in the HPA-axis in order to mobilize energy during stress. However, there is only very limited information about the effects of energy intake on the functioning of the HPA-axis in cattle.

The feeding level in terms of energy intake may affect the reactivity in the HPA-axis at specific levels. In order to study the effects of energy intake on the reactivity at different levels of the HPA-axis we measured the cortisol and ACTH responses to a CRF-challenge, an ACTH-challenge and to 15 minutes of social isolation in Holstein cows in early lactation (week 4-6 post partum) fed high (75 % concentrate) versus low (25 % concentrate) energy density in the diet from parturition. We found no differences in either ACTH or cortisol responses to intravenous injection of CRF or ACTH between the two levels of energy density (Munksgaard *et al*., 2003). Our results are supported by Fisher *et al*. (2002), who found no differences in cortisol or ACTH responses to iv injections of CRF when comparing lactating Jersey cows fed 3 or 4% of their body weight on a dry matter basis. Remarkably, we found that after exposure to 15 minutes of social isolation in an unfamiliar environment, the cows fed the high energy density diet had a lower cortisol response than the cows fed the low energy density diet (Munksgaard *et al*., 2003, Figure 1).

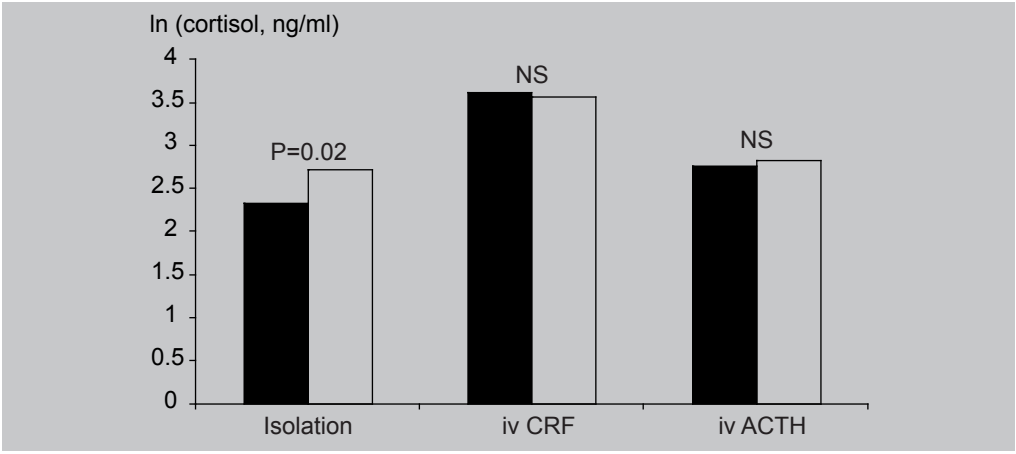

Figure 1. Maximum cortisol response of Holstein cows in early lactation fed a high energy density diet (■) or a low energy density diet (□), after social isolation for 15minuttes, iv injection of CRF or ACTH (Source: Munksgaard et al*., 2003).*

In humans and rodents consumption of glucose or a high carbohydrate diet can decrease the responses of the HPA-axis toward acute stress (Laugero, 2001, Dallman *et al*., 2003), suggesting that carbohydrate intake can modulate the responses of the HPA-axis. Although the underlying mechanisms have not yet been identified, there is evidence for the existence of a feed back system affecting CRF at the brain level, mediated by effects of glucocorticoids on metabolism. In adrenalectomized (ADX) rats it has been demonstrated that ingestion of sucrose, like replacement with corticosterone, normalise caloric intake, energy balance, secretion of ACTH and central expression of CRF (Bell *et al*., 2000, Laugero *et al*., 2001). Furthermore, in ADX-rats ingestion of sucrose led to increased plasma concentrations of leptin, insulin and triglyceride as well as reduced concentrations of NEFA. However, icv corticosterone did not restore feed intake or energy balance in ADX-rats (Laugero *et al*., 2002).

In our experiment the cows fed the high energy density in the diet had higher plasma concentrations of glucose and insulin, but lower NEFA and BHBA concentrations (Table 2). Although cows milked three times daily had lower plasma concentration of glucose and higher concentration of BHBA than cows milked two times a day, there were no differences in their plasma concentrations of insulin or NEFA or in the responses of the HPA-axis toward 15 minutes of social isolation between two and three times daily milkings (Andersen *et al*., 2004, Munksgaard *et al*., 2003). Thus our results give no support for a simple relation between baseline plasma concentrations of glucose and the reactivity of the HPA-axis at CNS level. In ADX-rats drinking sucrose the plasma

Table 2. Plasma metabolites, hormones and liver parameters in cows fed high versus low energy density in the diet and cows milked two versus three times a day (↑ indicate increased level in high compared to low or increased level in 2 compared to 3 times milking) (Source: Andersen et al*., 2002, 2003, 2004, Munksgaard* et al*., 2003).*

	High compared to Low energy density	**2 compared to 3 times milking a day**
Plasma level		
Cortisol	NS	NS
ACTH	NS	NS
Insulin	↑	NS
Glucose	↑	↑
BHBA	↓	↓
NEFA	↓	NS
Response to acute stress		
Cortisol, maximum response	↓	NS
Cortisol, area under curve	↓	NS
NEFA	NS	NS
Glucose	↑	NS
Liver parameters		
Long chain fatty acid oxidation capacity	↑	NS
Triacylglycerol content	↓	NS

concentration of glucose was not affected, despite sucrose drinking restored the activity of the HPA-axis and energy balance (Laugero *et al*., 2002).

Furthermore, in our experiment long chain fatty acid oxidation capacity in the liver was increased and liver triacylglycerol content was decreased in cows fed the high energy density compared with the low energy density diet, while there were no effects of milking frequency on liver parameters (Andersen *et al*., 2002, 2003). Thus our results are in agreement with results obtained in rats, suggesting that the metabolic state affects the regulation of CRF at the CNS level. This may be mediated trough metabolic hormones like insulin or regulations in the liver, whereas basal plasma concentration of glucose does not seem to be directly involved.

Diet composition may affect serotonin at CNS level

The brain serotonergic system is involved in the regulation of the activity of the HPA-axis (Fuller, 1996), and there is evidence that the activity of the serotonergic system relates closely to emotions like fear and anxiety as well as depressive mood (Koyama and Kusumi, 1995; Malmkvist *et al*., 2003). The amino acid tryptophan is precursor of brain serotonin, and diets lacking tryptophan can reduce brain serotonin levels (Yokogoshi, 1995). Circulating tryptophan can cross the blood-brain barrier, but circulating tryptophan competes with other large neutral amino acids for transport sites into the brain. Carbohydrate-rich, protein-poor diets can increase the ratio of tryptophan relative to the sum of other large neutral amino acids (Kishi *et al*.,1995, Markus *et al*., 2000), and such diets have in some studies in humans been reported to have positive effects on mood and to decrease cortisol and depressive mood after exposure to acute stress (Markus *et al*., 2000). In ruminants, the level of circulating tryptophan relative to other large neutral amino acids is less directly related to diet composition. However, Nakanishi *et al*., (1998) found, that oral administration of rumen protected tryptophan to three-month-old beef calves after weaning from their dams induced increased lying time and reduced agonistic interactions and vocalisations. Thus, feed rations resulting in different ratios of tryptophan to other large neutral amino acids must also be expected to affect central levels of serotonin in ruminants.

Can changes in the level of serotonin and CRF regulation at the CNS level affect behavioural responses to stress?

In humans and rodents there is evidence that carbohydrate intake has a modulatory effect on behavioural responses to stress (for review see Dallman *et al*., 2003, Laugero, 2001). This effect seems to be mediated by changes in the availability of trypthophan for central serotonin synthesis, and/or mediated by a metabolic pathway affecting CRF at the CNS level. However, there is also some evidence suggesting that the ratio between protein/carbohydrates in the diet can influence the regulation of dopamine receptors in the brain of rats (Hamdi *et al*., 1992*)*.

In our study, the cows fed a low energy density diet had increased latency to eat, decreased food intake and increased occurrence of self-grooming, when provided with novel food for 10 minutes compared to cows fed a high energy density diet (Herskin *et al*., 2003b). This might relate to the differences we observed in reactivity in the HPA-axis at CNS level in the same cows (Munksgaard *et al*., 2003). The brain CRF-system play a role in mediating behavioural responses to stress

(Koob, 1999), and intracerebral administration of CRF induce a number of behavioural effects like changes in locomotion, exploration and anxiety/fear related behaviour (for review see Dunn and Berridge, 1990, Weninger and Majzoub, 2001). Since both the central serotonergic and the CRF systems in the brain are related to emotional responses, differences in diet composition and energy density of the diet may change the emotional response to stressors and thereby affect the welfare of the animals.

How do diet composition and energy intake affect behaviour?

A straightforward example of an effect of food composition on behaviour is when animals are presented with novel food. Food neophobia, or the tendency to avoid novel food and require a period of habituation when food composition is suddenly changed, is well known in ruminants foraging in familiar environments (e.g. Chapple and Lynch, 1986, Burritt and Provenza, 1989, Provenza *et al*., 1995). Food intake can be close to zero on the first days after sudden food changes (Launchbaugh *et al*., 1997), and it can take up to 2 weeks before food intake is normalised (Chapple *et al*., 1987). Recently Herskin (2004) have focused on novel food as a potential stressor for dairy cows. Results of her studies demonstrate that dairy cows show strong behavioural responses when presented with novel food, including signs of motivational conflict and neophobia (Herskin *et al*., 2003a, b). However, the provision of novel food to dairy cows kept in tie stalls did not induce adrenocortical responses or increased heart rate, suggesting that novel food is a stressor of rather low intensity (Herskin, 2004).

There are numerous investigations on food selection, food preferences and feeding behaviour of cattle (as reviewed by Phillips, 2002, Forbes and Provenza, 2000), but it is beyond the scope of this paper to make a complete review of the entire topic. In this paper we focus on the effects of total energy intake and diet composition on time budgets, and discuss whether time restraints can have negative impact on dairy cows.

Effects of composition and energy density of the diet on time budgets

Like all other living creatures ruminants only have a total of 24 hours per day to allocate to different activities including eating behaviour. The composition of the diet, particularly the energy density, strongly affects the time needed for consuming any given amount of food. One kg of concentrate is typically consumed within 3 to 4 minutes while it takes about half an hour to eat one kg of hay (Phillips, 2002). Cows fed a low concentrate diet for ad libitum intake spend more time eating compared to cows fed rations with higher energy density (Nielsen *et al*., 2000, Munksgaard and Herskin, 2001, Figure 2). However, cows also spend time on other activities and the important questions are first whether cows have enough time for all high priority activities, and second how time constraints affects the animals.

Previous studies focusing on time budgets of dairy cows have shown that while eating time is increased on diets with low energy density compared to diets with a higher level of energy, lying time was decreased (Nielsen *et al*., 2000; Munksgaard and Herskin, 2001). Such changes in time budgets may reflect adaptation to a specific environment without having any negative

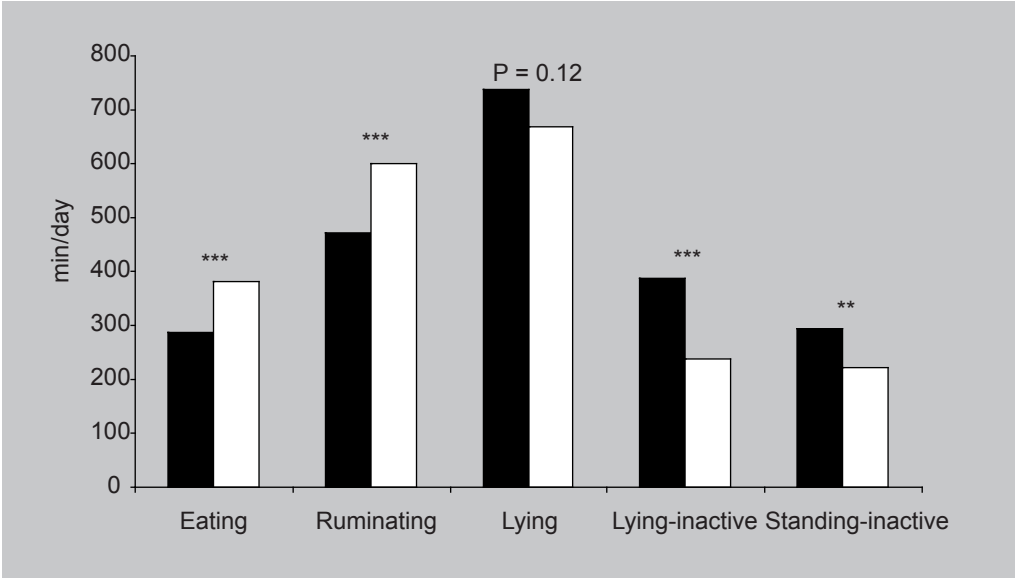

Figure 2. Time budgets of Holstein cows in early lactation feed a high energy density diet (■) and a low energy density diet (□) (Source: Munksgaard et al.*, 2005).*

consequences for the welfare of the animal, but if the constraint affects high priority behaviours, it may have negative consequences.

Repeated deprivation of lying induces behavioural as well as physiological stress responses (Munksgaard and Simonsen, 1996, Munksgaard *et al.*, 1999, Ingvartsen *et al.*, 1999, Fisher *et al.*, 2002). However, the exact need for lying time is not known and there is only limited information available about the relative priorities of different behaviours. Behavioural priorities can be quantified by estimation of behavioural demand functions by measuring how much the animals are willing to work for access to a given resource (price elasticity) (e.g. Dawkins, 1990; Matthews and Ladewig 1994) or by measuring the relative changes in the specific activities when the available time is limited (income elasticity) (e.g. Lea 1978; Dawkins 1983). When animals have less time available, it would be expected that relatively more time would be allocated to important behaviours while less or no time would be allocated to non-essential behaviours (Houston and McFarland, 1980).

Lying requirements of dairy cattle were studied in a series of experiments by estimating demand functions based on price elasticity. The results suggest that dairy heifers of about 400 kg have an inelastic demand for lying at about 12-13 hours per day (Jensen *et al.*, 2004). In another study, time constraints were used to estimate the relative priority between lying, eating and social behaviour; i.e. dairy cows were deprived of lying, eating and social contact for either 9 or 12 hours per day compared to a control treatment (Munksgaard *et al.*, 2005). Time constraint reduced the absolute amount of time spent on each of the behaviours measured. However, the proportion of time spent lying increased; the proportions of time eating and having social contact

remained similar to non-constrained cows. A comparison of the changes in the proportions of time spent on each activity suggests that the ranked priority for the various behaviours would be: first lying then eating followed by social contact (Figure 3). This is based on the assumption that compensatory mechanisms for each activity under time constraints are similar. However, food intake can be maintained under constraints by a variety of mechanisms (e.g. increased intake rate, increased bite size) whereas it is more difficult to imagine alternative mechanisms for maintaining rest or social contact under constraint. Previous studies have shown that both competition and restriction of the amount of food offered can increase the rate of feed intake in dairy cows (Nielsen, 1999). In the study by Munksgaard *et al.* (2005), the cows compensated for the time constraint by increasing the rate of feed intake, and therefore the reduction in feed intake was less than the reduction in eating time. However, it is remarkable that even though the cows increased the rate of feed intake, they did not completely compensate for the time constraint. In fact, the cows allocated relatively more time to lying at the expense of feed intake. This was not due to limitations in physical or metabolic capacity, since in another experiment using a similar total mixed ration cows that were not restricted in resting and social contact maintained feed intakes when restricted to 12 hours access to food (Munksgaard *et al.*, 2005). Thus, these results suggest that under time constraints, cows are willing to give up some feed intake in order to maintain lying time.

Furthermore, under commercial conditions in a loose housing system with cubicles (one cubicle per cow, one eating place per cow), we found a negative correlation between lying time and eating time and that lying time as well as time in cubicles was negatively correlated with yield in first lactation Holstein cows (Løvendahl and Munksgaard, 2004).

Based on the above results, as summarised in Figure 4, we suggest, that 1) the minimum need for lying time in dairy cows is at least 10 hours per day and that 2) cows to some degree will trade of eating time for lying time under time constraints.

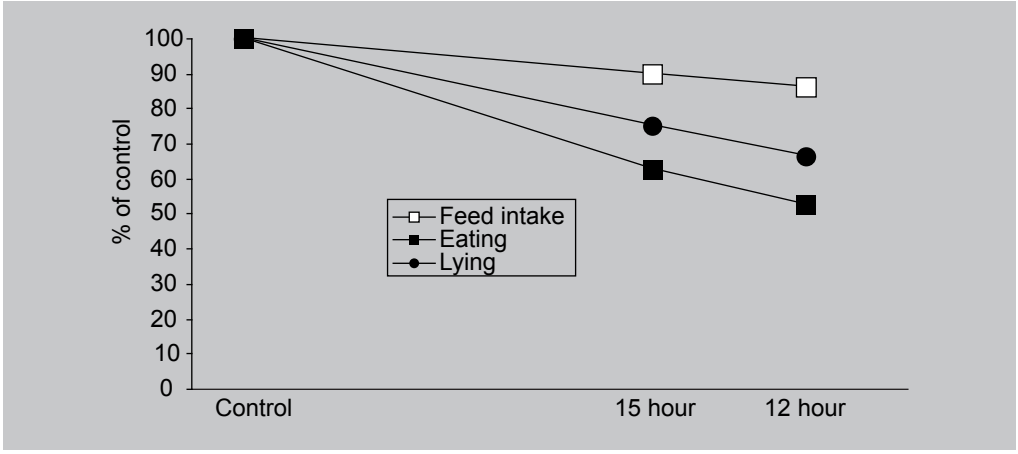

Figure 3. Lying time, eating time and feed intake (kg DM) as percentage of control treatment for cows restricted to 15 and 12 hours access to lying and eating (Source: Munksgaard et al.*, 2005).*

Figure 4. Estimated demand for lying in heifers and lying time in cows fed high and low energy density in the diet, under time constraint and lying time for cows in loose housing (Source: [a] Jensen et al.*, 2004, [b] Munksgaard and Herskin, 2001, [c] Munksgaard* et al.*, 2005, [d] Munksgaard and Simonsen, 1996, Løvendahl and Munksgaard, 2004).*

Therefore lack of time may be a problem in loose housing systems where cows have to spend time on other activities like walking between different resources and waiting for access to the milking parlour. This is especially true for high producing cows with a high demand for energy and thus a need for longer eating time. Furthermore, cows with a low position in the social hierarchy may have to spend extra time to get access to resources under crowded conditions. For instance, Ketelaar de Lauwere *et al.* (1996) found, that in a system with automatic milking the amount of time cows have to spend in the waiting area in front of the robot depended on their position in the social hierarchy. Thus, we suggest that under the present commercial conditions some cows may not be able to fulfil their needs for eating and lying time, and this may lead to a reduction in welfare as well as an increased risk of production diseases.

Conclusion

There is some evidence that nutrition in ruminants like in non-ruminant mammals can affect both the activity and reactivity of the HPA-axis as well as behavioural stress responses.

In ruminants feeding can induce a decrease and fasting can cause an increase in plasma concentrations of cortisol. Although there is some evidence that daily energy intake can affect the HPA-axis activity in heifers, results on lactating animals have not given support to such an effect. However, the total energy intake can affect both behavioural and HPA-axis reactivity at the CNS level toward acute stress in lactating cows. The effects of nutrition on reactivity to stress

needs to be investigated further in order to estimate the extent to which this has any detrimental effects under commercial conditions and in order to elucidate the mechanisms behind.

Low energy density in the diet can put dairy cows under time constraints due to a need for increased eating time in order to maintain feed intake. In dairy cows, eating time and lying time was negatively correlated, and it has been demonstrated that cows to some degree trade of eating time for lying time. Thus we suggest that some cows under commercial conditions may be in lack of time for fulfilling their needs for both eating and lying.

Acknowledgements

Thanks to Margit Bak Jensen for comments on earlier version of this manuscript. The Danish Villum Kann Rasmussen Foundation is acknowledged for the financial support to Mette S. Herskin.

References

Akana, S.F., A.M. Strack, E.S Hanson & M.F. Dallman (1994). Regulation of activity in the hypothalamo-pituitary-adrenal axis is integral to a larger hypothalamic system that determines caloric flow. Endocrinology 135, 1125-1134.

Andersen, J.B., N.C. Friggens, T. Larsen, M. Vestergaaard & K.L. Ingvartsen (2004). Effects of energy density in the diet and milking frequency on plasma metabolism and hormones in early lactating dairy cows. J. Vet. Med. A 51, 52-57.

Andersen, J.B., N.C. Friggens, K. Sejrsen, M.T. Sørensen, L. Munksgaard & K.L. Ingvartsen (2003). The effects of low vs. high concentrate level in the diet on performance in cows milked two or three times daily in early lactation. Livest. Prod. Sci. 81, 119-128.

Andersen, J.B., T. Larsen, M.O. Nielsen & K.L. Ingvartsen (2002). Effect of energy density in the diet and milking frequency on hepatic long chain fatty acid oxidation in early lactating dairy cows. J. Vet. Med. A 49, 177-183.

Beerda, B., J.E. Kornalijnslijper, J.T.N. van der Werf, E.N. Noordhuizen-Stassen & H. Hopster (2004). Effects of milk production capacity and metabolic status on HPA function in early postpartum dairy cows. J. Dairy Sci. 87, 2094–2102.

Bell, M.E., S. Bhatnagar, J. Liang, L. Soriano,T.R. Nagy & M.F. Dallman (2000). Voluntary sucrose ingestion, like corticosterone replacement, prevents the metabolic deficits of adrenalectomy. J. Neuroendocrinol. 12, 461-470.

Boissy, A. & M.F. Bouissou (1995). Assessment of individual differences in behavioural reactions of heifers exposed to various fear-eliciting situations. Appl. Anim. Behav. Sci. 46, 17-31.

Burrit, E.A. & F.D. Provenza (1989). Food aversion learning: ability of lambs to distinguish safe from harmful foods. J. Anim. Sci. 67, 1732-1739.

Chapple, R.S. & J.J. Lynch (1986). Behavioural factors modifying acceptance of supplementary foods by sheep. Res. Dev. Agr. 3, 113-120.

Chapple, R.S., M. Tomaszewska & J.J. Lynch (1987). The learning behaviour of sheep when introduced to wheat. 1. Wheat acceptance by sheep and the effect of trough familiarity. Appl. Anim. Behav. Sci. 18, 157-162.

Dallman, M. F., S.F. Akana, K.D. Laugero, F. Gomez, S. Manalo, M.E. Bell & S. Bhatnagar (2003). A spoonful of sugar: feedback signals of energy stores and corticosterone regulate responses to chronic stress. Phys. & Behav. 79, 3-12.

Dawkins, M.S. (1983). Battery hens name their price:consumer demand theory and the measurement of ethological 'needs'. Anim. Behav. 31, 1195-1205.

Dawkins, M.S. (1990). From an animal's point of view; motivation, fitness and animal welfare. Behaviour and Brain Science 13, 1-61.

Dunn, A.J. & C.W. Berridge (1990). Physiological and behavioural responses to corticotrophin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Res. Rev. 15, 71-100.

Fisher, A.D., M.A. Crowe, D.J. Prendiville, W.J. Enright (1997). Indoor space allowance, effects on growth, behaviour, adrenal and immune responses of finishing beef heifers. Anim. Sci.64, 53-62.

Fisher, A.D., G.A. Verkerk, C.J. Morrow & L.R. Matthews (2002). The effects of feed restriction and lying deprivation on pituitary-adrenal axis regulation in lactating cows. Livestock Prod. Sci. 73, 255-263.

Forbes, J.M. & F.D. Provenza (2000). Integration of learning and metabolic signals into a theory of dietary choice and food intake. In: Cronjé, P.B. (ed.) Ruminant physiology: digestion, metabolism, growth and reproduction. CABI Publishing CAB International, Wallingford UK. pp. 3-21.

Fuller, R.W. (1996). Serotonin receptors involved in regulation of pituitary-adrenocortical functioning in rats. Beh. Brain Res. 74, 215-219.

Gardy-Godillot, M., D. Durand, M. Dalle & D. Bauchart (1989). Diurnal pattern of plasma cortisol in preruminant calves fasted or fed different milk proteins. J. Dairy Sci. 72, 7, 1842-1846.

Hall, J.B., T.E Kiser, D.L. Boggs & R.S. Lowery (1991). Effects of dietary energy level on luteinizing hormone and adrenal function in the post partum beef cow. Theriogenology 36, 107-116.

Hamdi, A., E.S. Onaivi & C. Prasad (1992). A low protein-high carbohydrate diet decreases D2 dopamine receptor density in rat brain. Life Sciences 50, 1529-1534.

Herskin, M.S. (2004). Responses of dairy cattle toward acute stress and novelty – behaviour, physiology and nociception. Thesis. The Royal Veterinary and Agricultural University, Copenhagen. pp. 130.

Herskin, M.S., L. Munksgaard & A.M. Kristensen, A.M. (2003a). Testing responses to novelty in cattle: behavioural and physiological responses to novel food. Animal Science 76, 327-340

Herskin, M.S., L. Munksgaard & A.M. Kristensen (2003b). Behavioural and adrenocortical responses of dairy cows toward novel food: effects of food deprivation, milking frequency and energy density in the daily ration. Appl. Anim. Behav. Sci. 82, 251-265.

Houston, A.I. & D.J. Macfarland (1980). Behavioral resilience and its relation to demand functions. In: Limits to action: The allocation of individual behaviour (ed. J.E.R. Staddon.) Academic Press, New York, pp. 177-203.

Ingvartsen, K.L., L. Munksgaard, V.K.M. Nielsen & L.J. Pedersen (1999). Responses to repeated deprivation of lying down on feed intake, performance and blood hormone concentration in growing bulls. Acta Agric. Scand., Sect. A, Animal Sci. 49, 260-265.

Ingvartsen, K.L., R.J. Dewhurst & N.C. Friggens (2003). On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. Livest. Prod. Sci. 83, 277-308.

Jensen, M.B., L.J. Pedersen & L. Munksgaard (2004). The effect of reward duration on demand functions for rest in dairy heifers and lying requirements as measured by demand functions. Appl. Anim. Behav. Sci. (in press)

Johansson, B., I. Redbo & K. Svennersten-Sjaunja (1999). Effect of feeding before, during and after milking on dairy cow behaviour and the hormone cortisol. Anim. Sci. 68, 597-604.

Kazmer, G.W., M.A. Barnes & R.W. Canfield (1985). Reproductive and metabolic hormones during estrus after fasting in Holstein heifers. Theriogenology 24, 6, 619-629.

Ketelaar de Lauwere, C.C., S. Devir, J.H.M. Metz & C.C.K. de Lauwere (1996). The influence of social hierarchy on the time budget of cows and their visits to an automatic milking system. Appl. Anim. Behav. Sci. 49, 199-211.

Kishi, K., A. Yamauchi, F. Shizuka, Y. Kido, T. Nikawa & K. Rokutan (1995). Effect of macronutrients intakes on brain serotonin metabolite as measured by in vivo microdialysis in the rat. Serotonin in the central nervous system and periphery (ed: Takada, A. & Curson, G.). Int. Congress Series No. 1088, Elsevier, The Netherlands.pp 71-78.

Koob, G.F. (1999). Corticotropin-releasing factor, norepinephrine and stress. Biol. Psych. 46, 1167-1180.

Koyama, T. & I. Kusumi (1995). Role of serotonin (5-HT)2 receptor function: Implication for the etiology and treatment of depression. (ed: Takada, A. & Curson, G.). Int. Congress Series No. 1088, Elsevier The Netherlands. pp.121-127.

Ladewig, J. & D. Smidt (1989). Behavior, episodic secretion of cortisol, and adrenocortical reactivity in bulls subjected to tethering. Horm. & Behav. 23, 344-360.

Laugero, K.D. (2001). A new perspective on glucocorticoid feedback: Relation to stress, carbohydrate feeding and feeling better. J. Neuroendocrin. 13, 827-835.

Laugero, K.D., M.E. Bell, S. Bhatnagar, L. Soriano & M.F. Dallman (2001). Sucrose ingestion normalizes central expression of corticotrophin-releasing-factor messenger ribonucleic acid and energy balance in adrenalectomized rats: A glucocorticoid-metabolic-brain axis? Endocrin. 142, 7, 2796-2804.

Laugero**,** K.D., F. Gomez, S. Manalo & M.F. Dallman (2002). Corticosterone Infused Intracerebroventricularly Inhibits Energy Storage and Stimulates the Hypothalamo-Pituitary Axis in Adrenalectomized Rats Drinking Sucrose. Endocrin. 143, 4552 - 4562.

Launchbaugh, K.L., F.D. Provenza & M.J. Werkmeister (1997). Overcomming food neophobia in domestic ruminants through addition of familiar flavour and repeated exposure to novel foods. Appl. Anim. Behav. Sci. 54, 327-334.

Lea, S.E.G. (1978). The phsychology and economics of demand. Phsychological Bulletin 85**,** 441-466.

Løvendahl, P. & L. Munksgaard (2004). How can genetic variation in dairy cattle behaviour affect breeding and management decisions? Proc. XXI Genetic Days, Wroclaw, Poland. In: Animal Science Papers and Reports 22, 71-76.

Markus, R., G. Panhuysen, A. Tuiten & A. Koppeschaar (2000). Effects of food on cortisol and mood in vulnerable subjects under controllable and uncontrollable stress. Phys.& Beh. 70, 333-342.

Malmkvist, J., S.W. Hansen & B.M. Damgaard (2003). Effect of the serotonin agost buspirone on behaviour and hypothalamic-pituitary-adrenal axis in confident and fearful mink. Phys. & Beh. 78, 229-240.

Matthews, L. & J. Ladewig (1994). Environmental requirement of pigs measured by behavioural demand functions. Anim. Behav. 47**,** 713-719.

McCann, J.P. & W. Hansel (1986). Relationships between insulin and glucose metabolism and pituitary-ovarian functions in fasted heifers. Biol. of Reprod. 34, 630-641.

Mills, S.E. & B.F. Jenny (1979). Effects of high concentrate feeding and fasting on plasma glucocorticoids in dairy heifers. J. Anim. Sci. 49, 961-969.

Munksgaard, L., M.B. Jensen, L.J. Pedersen, S.W. Hansen & L.R. Matthews (2005). Quantifying behavioural priorities – effects of time constraints on behaviour of dairy cows, *Bos Taurus*. Appl. Anim. Behav. Sci. 92, 3-14**.**

Munksgaard, L., M.S. Herskin & P. Løvendahl (2003). Reactivity of the hypothalamo-pituitary-adrenal-axis in dairy cows - effects of frequency of milking and energy level in the feed ration. Proc. 37th International Congress of the ISAE, Abano Terme, Italy, p. 225.

Munksgaard, L. & M.S. Herskin (2001). Does milking frequency and energy concentration of the diet affect time budgets of high yielding dairy cows? Proc. 25th Congress of ISAE, 4-9 August, California, USA, 190.

Munksgaard, L., K.L. Ingvartsen, L.J. Pedersen & V.K.M Nielsen (1999). Deprivation of lying down affects behaviour and pituitary-adrenal axis responses in young bull. Acta Agric. Scand., Sect. A, Animal Sci. 49, 172-178.

Munksgaard, L. & H.B. Simonsen (1996). Behavioral and pituitary adrenal axis responses of dairy cows to social isolation and deprivation of lying down. J. Anim. Sci. 74, 769-779.

Moberg, G.P.(1985). Biological response to stress: key to assessment of animal well-being? In: Moberg, G.P. (ed.) Animal stress American Physiological Society, Bethesda, Maryland, pp. 27-49.

Nakanishi, Y., K. Shigemori, K. Yanagita & M. Manda (1998). Behaviuoral and growth effect of oral administration of rumen protected tryptophan. Mem. Fac. Agr. Kagoshima Univ. 34, 89-95.

Nielsen, B.L. (1999). On the interpretation of feeding behaviour measures and the use of feeding rate as an indicator of social constraint. Appl. Anim. Beh. Sci. 63, 79-91.

Nielsen, B.L., R.F. Veerkamp & A.B. Lawrence (2000). Effects of genotype, feed type and lactational stage on time budget of dairy cows. Acta Agric. Scand. Sec. A Anim. Sci. 50, 272-278.

Phillips, C. (2002). Cattle behaviour and welfare. Blackwell Science. pp.264.

Provenza, F.D., J.J. Lynch & C.D. Cheney (1995). Effects of a flavour and food restriction on the response of sheep to novel foods. Appl. Anim. Behav. Sci. 43, 83-89.

Rushen, J., A.M.B. de Passillé & L. Munksgaard (1999). Fear of people by cows and effects on milk yield, behavior and heart rate at milking. J. Dairy Sci. 82, 720-727.

Samuelsson, B., K. Uvnäs-Moberg, R. Gorewit & K. Svennersten-Sjaunja (1996). Profiles of the hormones somatostatin, gastrin, CCK, prolactin, growth hormone and cortisol. II. In dairy cows that are milked during food deprivation. Livest. Prod.Sci. 46, 57-64.

Sapolsky, R.M. (2002). Endocrinology of the stress-response. In: Becker, J.B., Breedlove, S.M., Crews, D. & McCarthy (eds.) Behavioral Endocrinology. A Bradford Book, The MIT Press (London) pp 409-451.

Schrick, F.N., J.C. Spitzer, T.C. Jenkins, D.M. Henricks & T.G. Althen (1990). Effetc of dietary energy restriction on metabolic and endocrine responses during the estrous cycle of the suckled beef cow. J. Anim. Sci. 68, 3313-3321.

Sejrsen, K., J.T. Huber & H.A. Tucker (1983). Influence of amount fed on hormone concentrations and their relationship to mammary growth in heifers. J. Dairy Sci. 66, 845-855.

Simonetta, G., D.W. Walker & I.C. McMillen (1991). Effect of feeding on the diurnal rhythm of plasma cortisol and adrenocorticotrophic hormone concentrations in the pregnant ewe and sheep fetus. Experimental Physiology 76, 219-229.

Strack, A.M., S.F. Akana, C.J. Horsley & M.F. Dallman (1997). A hypercaloric load induces thermogenesis but inhibits stress responses in the CNS and HPA system. American J. Physiol. 272, R840-848.

Trenkle, A & D.G. Topel (1978). Relationships of some endocrine measurements to growth and carcass composition of cattle. J. Anim. Sci. 46, 1604-1609.

Yokogoshi, H. (1995). Relationships between protein nutrition, neurotransmitter (serotonin) and memory-learning ability. Serotonin in the central nervous system and periphery (ed: Takada, A. & Curson, G.). Int. Congress Series No. 1088, Elsevier The Netherlands.pp61-70.

Ward, J.R., D.M. Henricks, T.C. Jenkins & W.C. Bridges (1992). Serum hormone and metabolite concentrations in fasted young bulls and steers. Dom. Anim. Endocrin. 9, 2, 97-103.

Weninger, S.C. & E.B Majzoub, (2001). Regulation and actions of corticotrophin-releasing hormone. In: Handbook of Physiology. Section 7: The endocrine system. Volume IV: Coping with the environment: Neural and endocrine mechanisms. McEwen, B.S. & Goodman, H.M.(eds.). Oxford University Press. pp.103-125.

Part VIII: Human health aspects

Milk fatty acids and human health: potential role of conjugated linoleic acid and *trans* fatty acids

D.E. Bauman[1], A.L. Lock[1], B.A. Corl[2], C. Ip[3], A.M. Salter[4] and P.W. Parodi[5]
[1] Cornell University, Department of Animal Science, Ithaca, NY, USA,
[2]Department of Dairy Science, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
[3]Department of Cancer Chemoprevention, Roswell Park Cancer Institute, Buffalo, NY, USA
[4]Division of Nutritional Biochemistry, University of Nottingham, Nottingham, United Kingdom
[5]Human Nutrition and Health Research, Dairy Australia, Melbourne, Australia

Abstract

Conjugated linoleic acid (CLA) and vaccenic acid (VA) are milk fatty acids that may benefit health maintenance. The major CLA isomer is *cis*-9, *trans*-11 and it originates mainly by endogenous synthesis from rumen-produced VA via mammary Δ^9-desaturase. Diet and differences among individuals are major factors affecting milk fat CLA. The protective effect of CLA against cancer is consistently observed in animal and in vitro studies, being especially effective in breast cancer models. VA is also anticarcinogenic through its conversion to CLA. Antiatherogenic properties of CLA have also been established in animal models, with effects due to both changes in lipoprotein and cholesterol metabolism and anti-inflammatory effects. Of special importance, naturally enriched VA/CLA butter has proven effective in cancer and arthrosclerosis models. Examinations of human applications are limited and problematic because these are chronic diseases with a lack of consensus biomarkers and there are difficulties in analyses of dietary VA/CLA. Furthermore, CLA and VA are *trans* fatty acids, and their potential beneficial effects on health appear to be in marked contrast to *trans* fatty acids derived from hydrogenated vegetable oils. Overall, dairy products enriched in VA/CLA represent functional foods that offer potential to benefit human health and disease prevention as well as improve public perception of milk.

Keywords: atherosclerosis, cancer, CLA, functional foods, milk fat, *trans* fatty acids, vaccenic acid

Introduction

Milk and dairy products were recognized as important foods as early as 4000 B.C. as evidenced by the depiction of dairying in rock drawings from the Sahara (Miller *et al*., 2004). The dairy industry has made continuous advancements over the years and today we have a wide variety of milks and dairy products available. These products make a significant contribution to our nutrient supply including energy, high quality protein and several key minerals and vitamins (Miller *et al*., 2004; National Research Council, 1988). With the projected growth in world population and the increased demand for animal-derived food products as living standards improve, dairy products will undoubtedly continue to be an important dietary source of nutrients.

Food provides essential nutrients, but there is also growing consumer recognition of the link between diet and health. This awareness impacts food choices and the phrase "functional food"

is a generic term often used to describe this concept (Milner, 1999). The National Academy of Science has defined functional foods as "any food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Institute of Medicine, 1994). Associated with increased consumer interest in the health benefits of foods, scientists are increasingly being asked to clarify the role of specific foods and food components in health maintenance and disease prevention. This has impacted research emphasis world-wide; for example, the recent National Academy of Sciences report on "Frontiers in Agricultural Research" identified research on bioactive food components as a key focus area to enhance human health through nutrition (National Research Council, 2003).

There are many diseases for which functional food components and health maintenance can play an important role. Cancer and coronary heart disease (CHD) are two examples, and epidemiological studies suggest that the risk for these diseases can be substantially reduced by diet modification. Functional food components from plants are often highlighted in popular press articles as the basis for lowered risk of these diseases from consumption of fruits and vegetables. Foods of animal origin also contain functional food components, but these have been less extensively investigated. In the case of dairy products, there has been a general perception that a food containing saturated fat is unlikely to be beneficial to health. Yet over the last decade, evidence has accumulated that the form of the dietary fat is very important in determining the relative risk to diseases like cancer and CHD, and that milk-derived fat may offer significant health benefits compared to some common sources of dietary fats (National Research Council, 1996; Parodi, 2004).

A number of components that possess anticarcinogenic and antiatherogenic properties have been identified in milk through the use of model systems. Several excellent reviews provide details on the specific investigations relating to these properties of milk components (Aimutis, 2004; Gill and Cross, 2000; Harper, 2001; Parodi, 1999; 2004; Vesper *et al*., 1999). The present review will focus on milk fat, and specifically on conjugated linoleic acid (CLA). CLA is among the most potent of the naturally occurring anticarcinogens and the level of intake that is effective in biomedical models appears attainable from natural foods. The National Academy of Sciences report on carcinogens and anticarcinogens in foods pointed out that "CLA is the only fatty acid shown unequivocally to inhibit carcinogenesis in experimental animals" (National Research Council, 1996). CLA also has antiatherogenic properties (Kritchevsky, 2003; Parodi, 2002) and this is an emerging area of research. In addition, vaccenic acid (*trans*-11 18:1; VA) is a precursor for the major CLA isomer in milk fat (see reviews by Bauman *et al*., 2003; Palmquist *et al*., 2005) and recent investigations have demonstrated that the VA in milk fat has anticarcinogenic and antiatherogenic properties (Lock and Bauman, 2004; Parodi, 2004). These unique biological effects of CLA and VA are related to their specific structure and the presence of a certain configuration of double bonds at specific positions in the fatty acid. For comparison, Figure 1 contrasts their structures with linoleic acid. In the following sections we will elaborate the state of knowledge for CLA as a functional food component; the discussion will also include VA because of its special relationship to CLA.

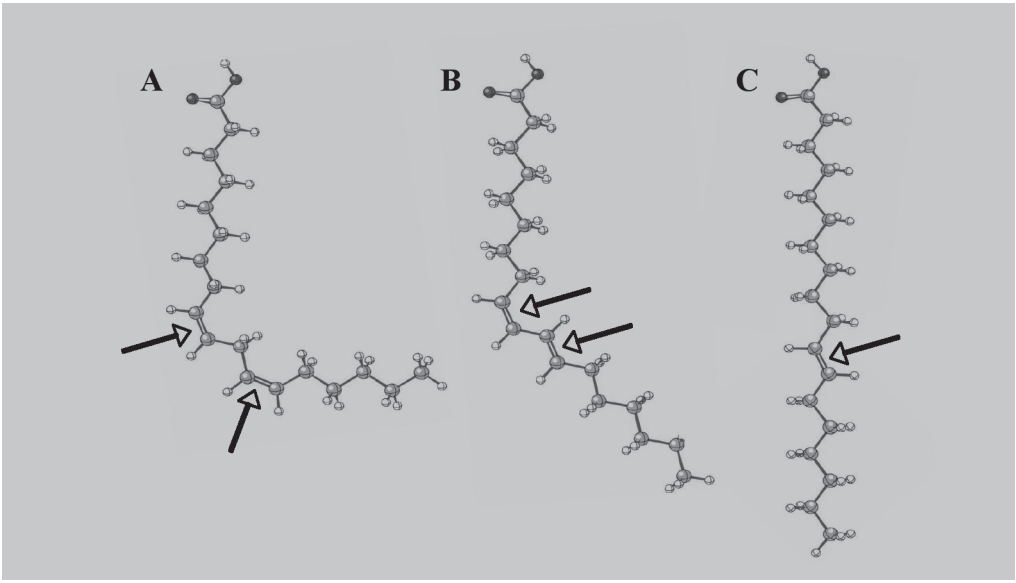

Figure 1. Comparison of the structures of linoleic acid (Panel A; cis-9, cis-12 18:2), cis-9, trans-11 conjugated linoleic acid (Panel B) and vaccenic acid (Panel C; trans-11 18:1). Double bonds are noted by arrows and structures are adapted from Bauman et al. *(2004).*

The biology of CLA

Background

The presence of milk fatty acids with a conjugated double bond system was first reported in the 1930's when increases in milk fat absorption at 230 nm were noted as cows shifted from winter confinement to spring pasture (Booth *et al*., 1933; Moore, 1939). Parodi (1977) was the first to demonstrate that the CLA in milk fat was mainly *cis*-9, *trans*-11 CLA, and the common name of "rumenic acid" has been proposed for this isomer because of its unique relationship to ruminants (Kramer *et al*., 1998). The discovery of a possible "functional food" role for CLA occurred in 1979 when Pariza and colleagues at the University of Wisconsin found that cooked beef contained a substance that inhibited mutagenesis (Pariza *et al*., 1979). They later found it consisted of isomers of conjugated octadecadienoic acid, for which they coined the name conjugated linoleic acids and the acronym CLA (see review by Pariza, 2004). Initial research on the biology of CLA focused on the anticarcinogenic effects, but as the research expanded, it was found that CLA had additional beneficial health-related effects including antiatherogenic, antiobesity, antidiabetogenic, and immunomodulation (see reviews by Belury, 2002; Pariza, 2004; Parodi, 2004). As a consequence, CLA research has increased exponentially over the last decade and most investigations have utilized synthetic preparations of CLA that contain a number of isomers in varied proportions. Only in the last few years have relatively pure isomers been experimentally used and, thus far, biological effects have only been extensively examined for two of them, *cis*-9, *trans*-11 and *trans*-10, *cis*-12.

Analytical advancements have allowed for a more complete characterization of CLA, and results indicate that ruminant fat contains numerous isomers; these differ by position or geometric orientation of the conjugated double bond pair and most are present at very low concentrations (see review by Lock and Bauman, 2004). However, *cis*-9, *trans*-11 is the major CLA isomer in ruminant fat, representing 75 to 90% of total CLA (Bauman *et al.*, 2000; Kraft *et al.*, 2003; Precht and Molkentin, 1999; Rickert *et al.*, 1999). Since *cis*-9, *trans*-11 is the predominant isomer by a substantial margin, enhancing milk fat content and functional food considerations of milk fat are realistically only related to this specific CLA isomer which has reported anticarcinogenic and antiatherogenic properties. Dietary supplements of *trans*-10, *cis*-12 CLA cause a reduction in body fat in a number of species, but doses required to elicit this effect are relatively high (Bauman *et al.*, 2003). Thus, food products from ruminants have little or no possibility of providing sufficient amounts of this isomer to have biological effects on body fat accretion; milk fat content of *trans*-10, *cis*-12 never represents more than 1 or 2% of total CLA (Lock and Bauman, 2004).

Dietary supply

The dietary source of CLA in humans has been examined for several countries and ruminant-derived food products are the major source (see review by Parodi, 2003). As illustrated by data from the US (Figure 2), dairy products account for about 70% of total food intake of CLA and ruminant meats account for another 25% (Ritzenthaler *et al.*, 2001). In general, processing conditions, storage and aging of various dairy products has little effect on CLA content. While a few studies have reported processing effects, they are of little consequence when compared to the variation in CLA content of ruminant fat that is related to diet, management and individual animal differences (see following section and reviews by Bauman *et al.*, 2003 and Lock and Bauman, 2004). Thus, the CLA content of ruminant-derived food products is related to the initial

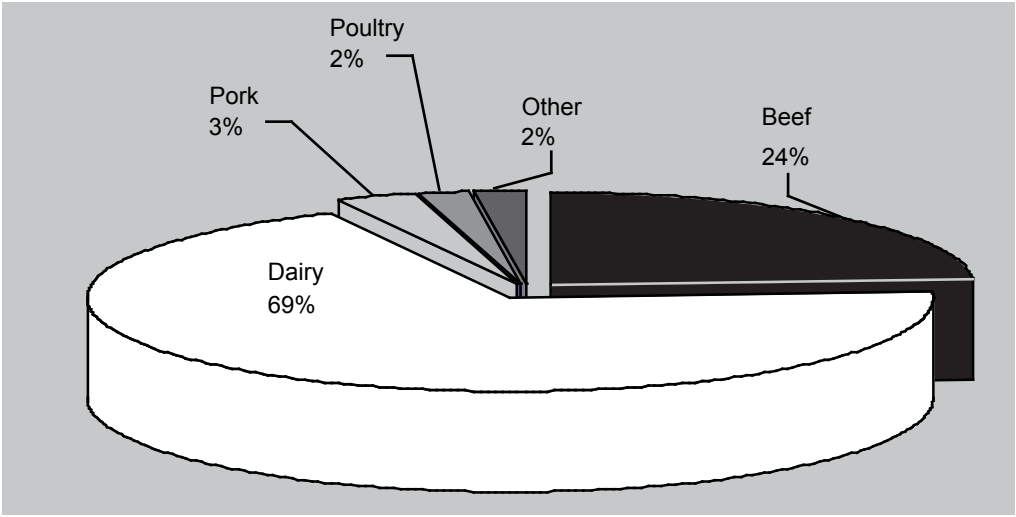

Figure 2. Contribution of different food sources to the dietary intake of CLA in the U.S. (Source: calculated from the data of Ritzenthaler et al. *(2001)).*

CLA content in milk and meat fat, and to the total fat content of the food product. The dietary intake of CLA has been examined for several countries and differences in food preferences result in a wide range in individual daily intakes. In general, the average dietary intake of CLA was in the range of 100 to 300 mg/d (see review by Parodi, 2003). However, this intake estimate does not include the contribution of endogenous synthesis of CLA from dietary VA. Based on kinetic data obtained from studies with humans (Turpeinen *et al*., 2002), Parodi (2003) suggested the dietary intake of CLA should be multiplied by 1.4 to obtain an estimate of the "effective CLA" provided by ruminant-derived food products.

Ruminant dimension

Rumen biohydrogenation

The presence of CLA in ruminant fats relates to the metabolism of dietary unsaturated fat by rumen microbes. The initial transformation that dietary lipids undergo in the rumen is hydrolysis of the ester linkages by microbial lipases (Dawson *et al*., 1977; Keeney, 1970). This step is a prerequisite for the second transformation, biohydrogenation of the unsaturated fatty acids. Rumen biohydrogenation was extensively investigated during the 1960's and 70's and these studies elaborated the major pathways (see reviews by Dawson and Kemp, 1970; Harfoot, 1981; Harfoot and Hazlewood, 1997; Keeney, 1970). Bacteria are largely responsible for biohydrogenation in the rumen and the first species identified was *Butyrivibrio fibrisolvens* (Kepler *et al*., 1966). As research efforts expanded, a range of rumen bacteria were shown to have the capacity to biohydrogenate unsaturated fatty acids, although most species are capable of only a portion of the sequence of reactions to convert PUFA to saturated fatty acids (see reviews by Harfoot and Hazlewood, 1997; Kemp and Lander, 1984; Palmquist *et al.*, 2005).

Linoleic acid (*cis*-9, *cis*-12 18:2) and α-linolenic acid (*cis*-9, *cis*-12, *cis*-15 18:3) are the predominant polyunsaturated fatty acids found in ruminant feedstuffs and major pathways for their biohydrogenation are shown in Figure 3. Isomerization of the *cis*-12 double bond is the initial step in the biohydrogenation of these fatty acids. For linoleic acid this isomerization produces *cis*-9, *trans*-11 CLA which in turn is reduced to VA and finally stearic acid. The initial isomerization of linolenic acid is also followed by a sequence of hydrogenations, and terminates with formation of stearic acid. However, it produces *cis*-9, *trans*-11, *cis*-15 conjugated octadecatrienoic acid as the initial isomerization product followed by hydrogenation of the remaining *cis*-double bonds. Therefore, VA is a common intermediate in the biohydrogenation of both α-linolenic acid and linoleic acid as well as in the biohydrogenation of γ-linolenic acid (*cis*-6, *cis*-9, *cis*-12 octadecatrienoic acid) (see reviews by Harfoot and Hazlewood, 1997; Palmquist *et al.*, 2005).

The CLA in milk fat was assumed to have originated predominately from CLA that escaped complete biohydrogenation in the rumen, but there were several inconsistencies with this idea. First, investigations of the kinetics of rumen biohydrogenation indicated that *cis*-9, *trans*-11 CLA was a transitory intermediate and VA was the intermediate that accumulated (see reviews by Griinari and Bauman, 1999; Harfoot and Hazlewood, 1997). Second, nutrition studies established that increases in the milk fat content of CLA occurred under dietary conditions that were unrelated to the supply of linoleic acid (see reviews by Bauman *et al*., 2001; Stanton *et al*., 2003).

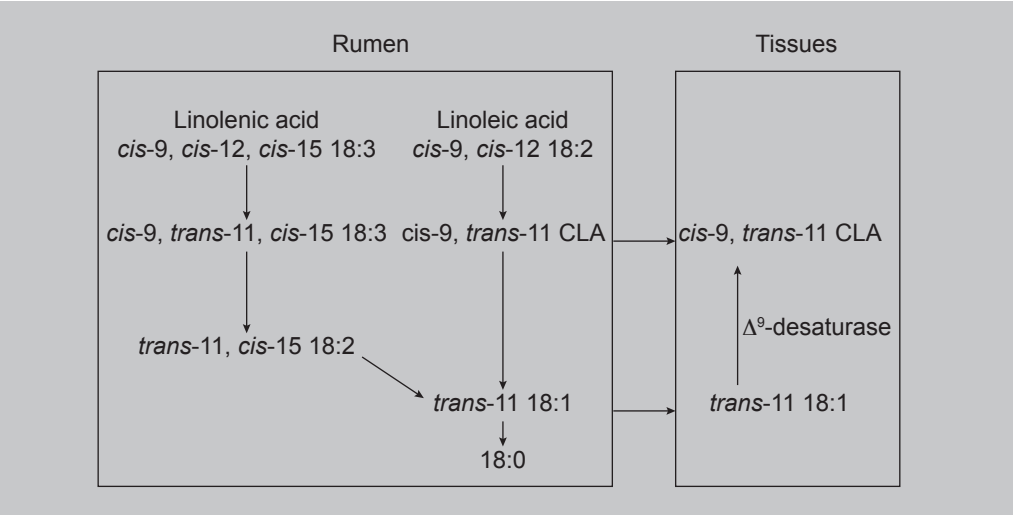

Figure 3. Pathways for rumen biohydrogenation and endogenous synthesis of cis-9, trans-11 CLA in ruminants (Source: adapted from Bauman et al. *(2003)).*

For example, dietary supplements of plant oils high in linolenic acid gave an increase in milk fat CLA (e.g. Dhiman *et al.*, 1999; Kelly *et al.*, 1998a; Lock and Garnsworthy, 2002), even though *cis*-9, *trans*-11 CLA is not an intermediate in the rumen biohydrogenation of linolenic acid. Based on these and other considerations, Griinari and Bauman (1999) proposed that endogenous synthesis could be an important source of the *cis*-9, *trans*-11 CLA found in milk fat, with synthesis involving the enzyme Δ^9-desaturase and VA as the substrate.

Endogenous synthesis

The first study to directly show that milk fat CLA could originate via endogenous synthesis was by Griinari *et al.* (2000) and involved abomasal infusion of VA; infusion of 12.5 g/d to lactating cows resulted in a 31% increase in milk fat content of *cis*-9, *trans*-11 CLA. This established the potential for endogenous synthesis of CLA, but the extent of its contribution was still unknown. Initial studies addressed this issue by inhibiting Δ^9-desaturase through abomasal infusion of sterculic oil. This oil contains two cyclopropenoic fatty acids, sterculic acid and malvalic acid, which are specific inhibitors of Δ^9-desaturase (Bickerstaffe and Johnson, 1972; Jeffcoat and Pollard, 1977; Phelps *et al.*, 1965). The initial investigation indicated that endogenous synthesis provided an estimated 64% of the *cis*-9, *trans*-11 CLA in milk fat (Griinari *et al.*, 2000), thereby establishing that endogenous synthesis was the major source of *cis*-9, *trans*-11 CLA in milk fat. Using the same approach, results were extended to other dietary situations and in all cases the majority of the *cis*-9, *trans*-11 CLA in milk fat was derived from endogenous synthesis via Δ^9-desaturase (Table 1). Pasture, which is high in linolenic acid, represents a dietary situation where milk fat content of CLA is markedly increased and it is noteworthy that endogenous synthesis accounted for over 91% of the total *cis*-9, *trans*-11 CLA in milk fat (Kay *et al.*, 2004).

Table 1. Evaluation of endogenous synthesis contribution to the cis-9, trans-11 CLA in milk fat from lactating dairy cows.

Diet and approach[1]	Milk fat CLA (mg/g fatty acid)	Endogenous synthesis estimate	Reference
Inhibition of Δ^9-desaturase[2]			
Hay/concentrate TMR	4	64%	Griinari *et al.* (2000)
Hay/concentrate TMR plus PHVO	7 8	78%	Corl *et al.* (2001)
Pasture	16	>91%	Kay *et al.* (2004)
Rumen Output[3]			
Grass silage/concentrate plus various plant oils	8-11	>80%	Lock and Garnsworthy (2002)
Corn silage/alfalfa hay TMR	5-7	>93%	Piperova *et al.* (2002)
Grass silage/concentrate plus fish oil	19	>74%	Shingfield *et al.* (2003)

[1]Diet abbreviations are TMR (total mixed ration) and PHVO (partially hydrogenated vegetable oil).
[2]Direct estimates of endogenous synthesis determined by abomasal infusion of sterculic oil as a source of cyclopropenoic fatty acids to inhibit Δ^9-desaturase.
[3]Indirect estimates determined by measuring rumen outflow of *cis*-9, *trans*-11 CLA and comparing this to the quantity of *cis*-9, *trans*-11 CLA secreted in milk fat.

A second approach to evaluate the source of *cis*-9, *trans*-11 CLA in milk fat was to estimate rumen outflow of this fatty acid (Table 1). By comparing this with milk output of CLA, the maximum proportion derived from rumen production can be estimated and endogenous synthesis would represent the remainder. For this indirect approach, representative samples of digesta were obtained and the CLA analysis data combined with marker-derived estimates of digesta flow rates. Lock and Garnsworthy (2002) conducted the first investigation with this approach, estimating that endogenous synthesis accounted for over 80% of the *cis*-9, *trans*-11 CLA in milk fat. Using a similar approach, Piperova *et al.* (2002) and Shingfield *et al.* (2003) reported that rumen outflow of *cis*-9, *trans*-11 CLA accounted for a maximum of 4 to 7% and 26% of the daily secretion of *cis*-9, *trans*-11 CLA in milk fat, respectively.

Overall, these investigations utilizing a range of diets and different experimental approaches reached a similar conclusion, the major source of *cis*-9, *trans*-11 CLA in milk fat is endogenous synthesis as depicted in Figure 3. Thus, the relatively constant milk fat ratio observed between VA and *cis*-9, *trans*-11 CLA reflects the substrate:product relationship for Δ^9-desaturase. The predominance of endogenous synthesis as the source of milk fat CLA highlights the critical role of Δ^9-desaturase in the biology of CLA.

Dietary and physiological effects

Diet is the major determinant of milk CLA content and numerous experiments have investigated methods to enhance milk fat CLA (see reviews by Bauman *et al*., 2001; Bessa *et al*., 2000; Chilliard *et al*., 2000; 2001; Stanton *et al*., 2003). Most investigations have involved lactating cows. However, the production of dairy products from goat and sheep milk is of economic importance in a number of countries and it appears conclusions derived with dairy cow studies also apply to these species. Increasing rumen VA outflow to provide precursor for endogenous synthesis is critical, and this can be achieved in two ways, increasing the supply of 18-carbon PUFA precursors and by inhibiting VA reduction to stearic acid. Increasing the dietary supply of 18-carbon PUFA substrates is most easily achieved by the addition of plant oils rich in linoleic and/or linolenic acids and a range of plant oils have been shown to be effective in increasing milk CLA content. The coat of oil seeds offers some protection from rumen biohydrogenation and thus processing of the seeds makes the oil accessible to biohydrogenating bacteria and results in greater increases in milk CLA compared with whole oil seeds. However, processed seeds are generally not as effective as using the pure oil (Chouinard *et al*., 2001; Dhiman *et al*., 2000; Stanton *et al*., 1997). There is a limit to the amount of lipid supplement that can be used to increase rumen outflow of VA and CLA. Ruminant diets are generally restricted to less than 7% fat because higher amounts adversely affect the metabolism of rumen bacteria, thereby impairing rumen fermentation and animal performance (Jenkins, 1993; Palmquist and Jenkins, 1980). Furthermore, under some conditions the rumen environment can be altered with plant oil supplementation so that a portion of the biohydrogenation intermediates produced are *trans*-10, *cis*-12 CLA and *trans*-10 18:1, and this leads to milk fat depression (see reviews by Bauman and Griinari, 2001; 2003).

Several dietary factors have been identified that increase milk fat CLA by altering the rumen environment resulting in an increase in rumen output of VA. Examples include alterations in the forage:concentrate ratio, dietary supplements of fish oil and restricted feeding (Bauman *et al*., 2001; Stanton *et al*., 2003). Fish oils are among the most effective of these (AbuGhazaleh *et al*., 2003; Offer *et al*., 1999; Shingfield *et al*., 2003; Whitlock *et al*., 2002) and because they supply minimal amounts of 18-carbon PUFA, the increases most likely result from a reduction in the conversion of VA to stearic acid as suggested by Griinari and Bauman (1999). This was recently demonstrated using *in vitro* cultures of mixed rumen bacteria incubated with linoleic acid where addition of docosahexaenoic acid (22:6; DHA) promoted VA accumulation (AbuGhazaleh and Jenkins, 2004).

Lastly, a combination of dietary supply of PUFA and modification of the rumen environment can be especially effective in increasing the CLA content of milk fat. Seasonal effects on milk CLA have been known for some time (Auldist *et al*., 2002; Banni *et al*., 1996; Lock and Garnsworthy, 2003; Riel, 1963) and these appear related to this. Fresh pasture results in a 2- to 3-fold increase in the CLA content of milk fat, but the effect diminishes as the pasture matures (Auldist *et al*., 2002; Kelly *et al*., 1998b; Lock and Garnsworthy, 2003; Stanton *et al*., 1997). These results cannot be totally explained in terms of the fatty acid composition and supply of PUFA that grass provides; therefore, there must be additional factors or components of grass that promote the production of VA in the rumen, and these lessen in effect as the pasture matures. Likewise, the general trend is that feeding dietary supplements of fish oil together with plant oils results in a greater increase

in milk CLA content than feeding plant oils alone (eg. AbuGhazaleh *et al*., 2003; Whitlock *et al*., 2002).

Surveys have demonstrated an 8-fold range among herds in milk fat content of CLA with diet accounting for the majority of these differences (see reviews by Bauman *et al*., 2001; Bessa *et al*., 2000; Jahreis *et al*., 1999). However, a 2- to 3-fold variation in milk fat content of CLA among individual cows within a herd has also been reported (e.g. Kelly *et al*., 1998a; 1998b; Lawless *et al*., 1998; Lock and Garnsworthy, 2002, 2003; Peterson *et al*., 2002). A similar level of variation also occurs in the milk fat desaturase index, with a several-fold range among cows (Kelsey *et al*., 2003; Lock and Garnsworthy, 2002; 2003; Peterson *et al*., 2002). This index is calculated using fatty acid pairs that are substrate/product for Δ^9-desaturase and is a proxy for the enzyme (see discussion in Kelsey *et al*., 2003). Differences in desaturase index appear to have a genetic basis, although this has not been examined rigorously. Cows maintain a consistent hierarchy in milk fat content of CLA and desaturase index over time when fed the same diet and when switched between diets (Peterson *et al*., 2002). There were no breed differences (Holstein vs Brown Swiss) in a large study (>200 cows) by Kelsey *et al.* (2003). Although some have proposed breed differences in CLA content of milk fat (Lawless *et al*., 1999; White *et al*., 2001; Whitlock *et al*., 2002), these studies have generally involved very few animals and comparisons were sometimes confounded by diet. Thus, if breed differences do exist, they must be minor compared with the effect of diet and the variation among individuals (Bauman *et al*., 2003; Palmquist *et al*., 2005). Differences in milk CLA among cows are presumably related to individual variation in expression of the Δ^9-desaturase gene and rumen outflow of VA and CLA. Examination of other physiological factors has established that milk fat content of CLA and desaturase index have little relation to milk yield, milk fat yield, parity or stage of lactation (Kelsey *et al*., 2003; Lock *et al*., 2005a). Thus, by using a combination of diet formulation and selection of high individuals, a substantial enrichment of the milk fat CLA and VA can be achieved (Table 2).

Milk quality considerations

CLA-enriched dairy products have been evaluated for taste, organoleptic properties and storage characteristics in comparisons with standard dairy products (Baer *et al*., 2001; Ramaswamy *et al*., 2001a, b). Off-flavors due to fatty acid oxidation are of prime concern given the greater unsaturation of the milk fat. Results of the above investigations indicated that milk quality was unaffected, but in most cases the enrichment of milk fat CLA was modest. Lynch *et al.* (2005) recently evaluated milk that was naturally enriched with about 8- to 10-fold higher contents of CLA and VA (47 and 121 mg/g fatty acids, respectively). There was no difference in flavor or susceptibility to oxidation between 2% pasteurized milks with and without elevated levels of CLA and VA either initially or over a 14-day shelf-life (Lynch *et al*., 2005). Thus, milk and other dairy products enriched in CLA and VA appear to present no unique challenges related to product quality.

Table 2. Fatty acid composition of standard butter fat and high vaccenic acid/conjugated linoleic acid (VA/CLA) butter fat used in animal model studies of human health (Corl et al.*, 2003; Lock* et al.*, 2004).*

Fatty acid	Standard butter	VA/CLA butter
	g/100 g fatty acids	
4:0	4.12	3.69
6:0	2.04	1.81
8:0	1.10	0.99
10:0	2.27	2.10
12:0	2.50	2.31
14:0	8.87	8.67
14:1, *cis*-9	0.74	0.63
15:0	0.75	0.82
16:0	27.62	23.01
16:1, *cis*-9	1.52	1.33
17:0	0.47	0.45
18:0	11.85	5.09
18:1, *trans*-6 to 8	0.46	1.21
18:1, *trans*-9	0.36	0.84
18:1, *trans*-10	0.67	2.68
18:1, *trans*-11	1.30	16.28
18:1, *trans*-12	0.77	2.48
18:1, *cis*-9	25.35	14.23
18:2, *cis*-9, *cis*-12	3.33	2.93
CLA, *cis*-9, *trans*-11	0.51	3.76
18:3, *cis*-9, *cis*-12, *cis*-15	0.41	0.46
20:0	0.14	0.13
Others	2.85	4.10

Use of models to investigate effects of CLA on disease

Cancer models

There are biomedical models for most types of cancer and many of these have been used to investigate the role of CLA as an anticarcinogen (see reviews by Banni *et al.*, 2003; Belury, 2002; Ip *et al.*, 2003; Parodi, 2004; Scimeca, 1999). One model is the use of cell lines where physiological concentrations of CLA have suppressed proliferation in a wide range of human cancer cells including cell lines derived from breast, ovary, prostate, colon, liver, lung, melanoma, mesothelioma, glioblastoma, and leukemia. Transplanting tumor cells is another model and dietary supplements of CLA were effective in reducing the size and metastasis of transplanted human breast cancer cells and prostrate cancer cells in SCID mice. *In situ* organ site carcinogenesis models are of particular value in cancer investigations and dietary supplements of CLA were effective in inhibiting chemically induced skin papillomas, forestomach neoplasia, and preneoplastic lesions and tumors in the colon and mammary glands.

The anticarcinogenic effect of CLA is particularly impressive in studies of chemically induced mammary cancer; Ip and coworkers have used the rat mammary model and their contributions have been of special significance. They found that the dietary intake of CLA gives a dose-dependent reduction in the incidence and number of tumors (Ip *et al*., 1991; 1994). This is particularly noteworthy because studies with animal models routinely administer very high doses of carcinogen to experimentally achieve a multitude of tumors in all animals over a short time interval. However, a dose-response relationship was evident even at low carcinogen doses and tumor development was inhibited by CLA at levels as low as 0.05% of the diet (Ip *et al*., 1994). The anticancer effects of CLA in the rat mammary model were independent of the type or level of fat in the diet (Ip *et al*., 1991; 1996). Ip and coworkers also demonstrated that feeding CLA during the weaning to peripubertal period offered protection against mammary tumor development even when the carcinogen was administered at a later time (Thompson *et al*., 1997). On the other hand, when rats received no CLA supplementation until they were older and had mature mammary glands, the protective effect was achieved only when CLA was fed continuously during the tumor promotion period following the administration of the carcinogen (Ip *et al*., 1995). This response and the general observation that CLA is especially effective in the mammary gland may relate to the fact that the mammary tissue of most species consists largely of adipocytes. CLA is incorporated into triglycerides and phospholipids, but to a much greater extent in the triglyceride fraction (Banni *et al*., 2001). Thus, CLA would preferentially accumulate in the mammary adipocytes during the development of the glands, and this may represent a reservoir of CLA that offers protection from carcinogens in later life.

The CLA preparation used for most studies of experimentally induced cancer consisted of a mixture of isomers synthetically produced from vegetable oils. Although *cis*-9, *trans*-11 and *trans*-10, *cis*-12 were the most abundant, the synthetic mixtures generally contain at least a dozen CLA isomers (Banni *et al*., 2003; Saebø, 2003). In addition, CLA isomers in the synthetic preparations were in the form of free fatty acids. Thus, dietary supplements provided as capsules containing the synthetic preparation of CLA differ in both form and isomer profile from the CLA supplied as a functional food component present in dairy products.

A functional food approach to provide CLA was evaluated in a series of investigations that used a naturally enriched CLA butter as the food source and the rat prepubertal mammary cancer model. Feeding dairy cows a typical commercial diet supplemented with plant and fish oils allowed for the production of high CLA milk fat which was converted into butter as a convenient food source; the CLA content of the fat approached 4% of total fatty acids and *cis*-9, *trans*-11 represented 90% of the total CLA (Bauman *et al*., 2000). Results from the initial investigation established that *cis*-9, *trans*-11 CLA was an effective anticarcinogen when it was supplied as a dietary food in a natural form (CLA esterified in milk fat triglycerides) (Table 3; Ip *et al*., 1999). Interestingly, tissue concentrations of *cis*-9, *trans*-11 CLA were greater in rats fed the CLA-enriched butter than for rats fed a comparable amount of chemically synthesized *cis*-9, *trans*-11 CLA. As discussed earlier, the majority of the CLA in milk fat is synthesized endogenously from VA, and, as a consequence, the milk fat content of VA and CLA generally approximate a 3:1 ratio and change in concert (Bauman *et al*., 2003). Thus, the enriched butter was higher in both CLA and VA. In subsequent investigations we established that VA was anti-carcinogenic and that dietary VA resulted in a dose-related decrease in the risk of premalignant lesions or mammary

Table 3. Bioassay of mammary cancer prevention in rats fed different sources of CLA.[1]

Dietary treatment	Total CLA in diet (%)	CLA content (µg/mg lipid)		Mammary tumors	
		Plasma	Mammary fat	Incidence	Total No.
Control butter	0.1	5.4 [a]	7.2 [a]	28/30 [a] (93%)	92 [a]
High VA/CLA butter	0.8	23.3 [b]	36.5 [c]	15/30 [b] (50%)	43 [b]
Control butter and synthetic CLA	0.8	18.4 [b]	26.2 [b]	16/30 [b] (53%)	46 [b]

[1]Dietary treatments were initiated at weaning and continued for 30 days. All animals were then injected with methylnitrosurea (MNU) to induce mammary tumors and switched to a 5% corn oil diet with no CLA. They remained on this diet for 24 weeks and were then killed for tissue analysis. Values with unlike superscripts in the same column (a,b,c) differ $P < 0.05$. Adapted from Ip *et al.* (1999).

tumorgenesis whether VA was supplied as a synthetic chemical (Banni *et al.*, 2001) or as a natural component of VA/CLA enriched dairy products (Corl *et al.*, 2003). Dietary supplements of VA also resulted in a parallel dose-related increase in tissue content of CLA, so it was of obvious interest whether the anticarcinogenic properties of VA were direct or mediated by its conversion to *cis*-9, *trans*-11 CLA. We postulated the latter and investigated this using sterculic oil as a source of cyclopropenoic fatty acids to inhibit Δ^9-desaturase (Lock *et al.*, 2004). The addition of sterculic oil to the diet markedly attenuated the biological effects of VA on tissue content of CLA and the number and growth of preneoplastic lesions (Table 4). Thus, the anticarcinogenic effects of VA are predominately, perhaps exclusively, mediated through its conversion to *cis*-9, *trans*-11 CLA via Δ^9-desaturase. Overall, this vanguard series of pre-clinical investigations demonstrated the feasibility of the functional food approach using VA/CLA enhanced dairy products in the prevention of mammary cancer.

Establishing the mechanism for the anticarcinogenic effects of CLA is an active area of research. We will only provide a general overview with example citations, and would refer readers to several excellent reviews for a detailed discussion of potential mechanisms (Banni *et al.*, 2003; Belury, 2002; Ip *et al.*, 2003). Premalignant lesions and tumors grow when the rate of cell proliferation exceeds cell death, and results indicate that the anticancer properties of CLA may involve several mechanisms. In various cancer models, CLA has been shown to decrease cell proliferation as well as enhance rates of apoptosis in premalignant lesions and tumors (see Ip *et al.*, 2001, 2003). Other studies have established that CLA inhibits angiogenesis in some model systems thereby limiting the development of new blood vessels that are required for the growth and spread of tumors (Masso-Welch *et al.*, 2002). The anticarcinogenic effect of CLA has been observed using both direct and indirect acting carcinogens, confirming that the mechanism by which it acts in the mammary gland is not a result of inhibition of carcinogen activation (Ip *et al.*, 1994, 1995). There is also evidence that part of the effects could be mediated by the mammary stroma and modulation of the immune cell environment by CLA (Ip *et al.*, 2003), and early investigations also suggested the mechanism could involve CLA as an antioxidant (Ip *et al.*, 1991). Eicosanoids may

Table 4. Anticarcinogenic effect of vaccenic acid (VA) in dairy fat is mediated through its conversion to cis-9, trans-11 CLA via Δ^9-desaturase.[1]

Dietary Treatment[2]		CLA Content (µg/mg lipid)		Mammary premalignant lesions (n)
VA (%)	Sterculic oil	Plasma	Mammary fat	
0.13	-	11.3[a]	21.3[a]	83
0.13	+	13.0[a]	21.4[a]	80
1.60	-	31.3[c]	47.5[c]	43[*]
1.60	+	18.9[b]	28.9[b]	68

[1]Female rats were injected with a single dose of carcinogen (MNU) and then fed 1 of 4 diets. After 6 weeks, animals were killed and samples were evaluated. Adapted from Lock *et al.* (2004).
[2]All diets contained 10% butter that was provided as standard butter (low VA diets) or VA/CLA-enriched butter (high VA diets). Synthetic *cis*-9, *trans*-11 CLA was added to the standard butter diets so that the CLA content equaled 0.32% for all diets. To inhibit Δ^9-desaturase, sterculic oil was added where indicated at 0.40% of the diet and this provided cyclopropenoic fatty acids at 0.25% of the diet. For CLA content different superscripts in a column denote statistical differences ($P<0.05$). For mammary premalignant lesions, the asterix denotes significant difference from other treatment groups as evaluated by Poisson regression.

mediate some of the anticancer effects of CLA because novel conjugated fatty acids are formed as intermediates when CLA is metabolized through elongation and desaturation (Banni *et al.*, 2003). These processes are in common with other PUFA and as a result dietary CLA causes alterations in eicosanoid synthesis through inhibitory effects on lipoxygenase and cyclooxygenase in some cancer models (see Belury 2002; Banni *et al.*, 2003).

There is support for a number of different molecular mechanisms as the basis for the anti-carcinogenic effects of CLA. One of these is the peroxisome proliferator-activated receptor (PPAR) family of transcription factors and both *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA are ligands for PPAR- α and PPAR- γ. These CLA isomers have also been shown to activate the translation activity of PPAR-α, PPAR-β, and PPAR-γ (Ip *et al.*, 2003; Belury *et al.*, 2002). All three of these PPARs are expressed in the mammary gland, so one or more members of this transcription factor family may mediate the anticarcinogenic effect of CLA. Reduced levels of cyclin D1 and cyclin A were associated with the CLA-induced decrease in proliferation and changes in VEGF and flk-1 appear to mediate, at least in part, the inhibitory effect of CLA on angiogenesis (Ip *et al.*, 2001; Masso-Welch *et al.*, 2002). Still other research with various tissues and cell lines has implicated a role for the local production of other factors in mediating the CLA response including leptin, IGF-1, and IGF-2 (Ip *et al.*, 2003). On the whole, investigations to date suggest that the anticarcinogenic effect of CLA involves a multitude of mechanisms, and these may vary in importance depending on the tissue-specific process being regulated. The opportunity to exploit this diversity in CLA mechanisms may form the basis for the range in tissues and cancer types in which CLA is effective.

Atherosclerosis models

By comparison to the extensive studies on anticarcinogenic properties of CLA, investigations of the effects on atherosclerosis are limited. A number of animal models have been shown to develop atherosclerosis when fed an appropriate diet and these include rabbits, hamsters and mice. In general, these models respond to a diet rich in saturated fat and cholesterol with increases in plasma concentrations of cholesterol and/or triacylglycerol. This can lead to the development of atherosclerotic lesions in the aorta that exhibit similarity to early lesions seen in the arteries of humans. However, the relatively high concentration of dietary cholesterol required to induce lesions often makes it difficult to use these models to elucidate the effects of other dietary components. More recently, the development of transgenic strains of mice which spontaneously develop atherosclerosis or require much lower amounts of dietary cholesterol have proved useful additional models. It is important to note that most animal models develop atherosclerosis in response to raised plasma lipid levels and it is clear that this is only one of a wide range of risk factors that contribute to the clinical manifestations of cardiovascular disease in humans (Lusis, 2000).

A number of animal studies have demonstrated that dietary supplementation with mixtures of CLA isomers can reduce the development of atherosclerotic lesions. Early studies in rabbits, fed a high fat, high cholesterol diet, showed a significant reduction in aortic atherosclerosis after 22 weeks in animals fed 0.5 g CLA/day (Lee *et al*., 1994). Subsequent work indicated that dietary CLA could even induce the regression of pre-existing lesions in rabbits (Kritchevsky *et al*., 2000). This was particularly remarkable as CLA appeared to actually increase plasma cholesterol and triacylglycerol, suggesting the mechanism was independent of changes in plasma lipids. Recently, investigations utilizing pure isomers have demonstrated that *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA are equally effective in reducing cholesterol-induced atherogenesis in rabbits (Kritchevsky *et al.*, 2004). In hamsters, synthetic CLA isomer mixtures inhibited the development of cholesterol-induced atherosclerosis to a similar extent to linoleic acid when fed a diet containing 10% saturated fat (Nicolosi *et al*., 1997), but were more effective when the saturated fatty acid content of the diet was increased to 20% (Wilson *et al*., 2000). Few published studies have examined the effect of CLA on the development of atherosclerosis in the mouse. However, one such study, in the atherosclerosis susceptible C57BL/6 mouse, reported that CLA had no effect on, or could even promote atherosclerotic lesion development (Munday et al., 1999). More recently Toomey *et al.* (2003) reported that *cis*-9, *trans*-11 CLA induced regression of atherosclerosis in ApoE$^{-/-}$ knockout mice. This transgenic model has been widely used in studies of atherosclerosis and spontaneously develops lesions on a regular low-fat, low-cholesterol diet with a similar histopathology to lesions that develop in humans (Meir and Leitersdorf, 2004). Thus, the ability of *cis*-9, *trans*-11 CLA to induce a regression in atherosclerotic lesions in this animal model is of particular significance.

In addition to atherosclerosis end points, a number of recent studies have specifically investigated the effects of CLA on cholesterol and lipoprotein metabolism (Table 5). The male Golden Syrian hamster has been a valuable model for studying nutritional influences on cholesterol and lipoprotein metabolism because its lipoprotein system is more similar to humans than other rodent species (Spady and Dietschy, 1983; Salter *et al*., 1998; Billett *et al*., 2000). In this model,

animals supplemented with a synthetic mixture of CLA isomers had significantly lower plasma cholesterol and triacylglycerol (de Deckere *et al*., 1999; Gavino *et al*., 2000; Sher *et al*., 2003; Yeung *et al*., 2000). Studies specifically investigating the effects of pure isomers yielded conflicting results with one study (Valeille *et al*., 2004) showing that *cis*-9, *trans*-11 CLA was effective in lowering plasma cholesterol levels, but others (de Deckere *et al*., 1999; Gavino *et al*., 2000) indicate that this isomer had no effect. To date, all of the studies investigating the effects of CLA on atherosclerosis, cholesterol and lipoprotein metabolism have involved synthetic sources of CLA. We recently completed a study to examine the functional food potential of CLA in which Golden Syrian hamsters were fed a high fat (20%), high cholesterol (0.2%) diet containing standard or VA/CLA-enriched butter. The VA/CLA-enriched butter had a number of beneficial effects including reduced total and pro-atherogenic plasma cholesterol levels (Lock *et al*., 2005b). As discussed earlier, dairy products also contain VA and this would also contribute to tissue *cis*-9, *trans*-11 CLA through endogenous synthesis. It is of note that the VA/CLA-enriched butter produced a less atherogenic profile than an equivalent diet in which VA/CLA were replaced with *trans* fatty acids from hydrogenated vegetable oil (Lock *et al*., 2005b). These data illustrate the variety of results obtained in the hamster model so far (Table 5). Reasons for such variability in response are most likely a combination of effects of background diet, CLA isomer mix, age and strain of animals and length of study. Mechanisms for the effects of CLA on cholesterol and lipoprotein metabolism are not well understood, but are presumably related to effects on hepatic cholesterol metabolism. Supplementation with a VA/CLA-enriched butter reduced plasma concentrations of VLDL and LDL cholesterol lipoproteins suggesting that CLA may modify the production of atherogenic lipoproteins by the liver (Lock *et al*., 2005b). The effects of CLA on hepatic lipoprotein metabolism have recently been reviewed, and consistent with results above, the authors proposed that *cis*-9, *trans*-11 CLA could, in the absence of other CLA isomers, improve hepatic lipid metabolism (McLeod *et al*., 2004). This may explain the fact that VA/CLA-enriched butter elicited such impressive effects compared to when CLA isomer mixtures are used, since naturally-derived sources of CLA realistically only provide *cis*-9, *trans*-11 CLA.

As described above, at least a part of the beneficial effects of CLA on the development of early atherosclerotic lesions may be the result of modification of cholesterol and lipoprotein metabolism. However, in other studies, the reduced incidence of atherosclerosis in animals fed CLA was not accompanied by an improvement in the plasma lipid profile during the CLA feeding phase (Kritchevsky *et al*., 2000; 2002; Wilson *et al*., 2000). Atherosclerosis can also be considered as a chronic inflammatory disease and *cis*-9, *trans*-11 CLA has been shown to exhibit a number of important anti-inflammatory properties as well. These include a reduction in the expression of COX-2, PGE_2, reduced release of nitric oxide and a decreased production of pro-inflammatory cytokines (Urquhart *et al*., 2002). As mentioned earlier, *cis*-9, *trans*-11 CLA is a potent activator of PPARs, with the inhibition of pro-inflammatory products by *cis*-9, *trans*-11 CLA associated with PPARγ activation (Yu *et al*., 2002). Furthermore, the regression of pre-established atherosclerosis in $ApoE^{-/-}$ mice fed *cis*-9, *trans*-11 CLA observed by Toomey *et al.* (2003) was associated with an increased expression of PPARγ in the aorta. Research into these areas will undoubtedly aid our understanding of the mechanisms of action of CLA on atherosclerosis. Nevertheless, the overall results indicate that the predominant CLA isomer in dairy products, *cis*-9, *trans*-11, has beneficial effects in reducing risk factors for atherosclerosis, thereby demonstrating a clear potential for a functional food application.

Table 5. Effect of conjugated linoleic acid on lipoprotein cholesterol concentrations in the Golden Syrian Hamster.

Study	CLA composition	Cholesterol		
		Total	LDL	HDL
Nicolosi *et al.* (1997)	mixed	↓	↓	→
de Deckere *et al.* (1999)*	mixed	→	↓	↓
	c9, t11	→	n.r.	→
	t10, c12	→	↓	↓
Gavino *et al.* (2000)	mixed	↓	↓	→
	c9, t11	→	→	→
Yeung *et al.* (2000)	mixed	→	n.r.	→
Sher *et al.* (2003)	mixed	↓	n.r.	n.r.
Navarro *et al.* (2003)	c9, t11	→	→	→
	t10, c12	↓	↓	→
Valeille *et al.* (2004)	mixed	→	n.r.	→
	c9, t11	↑	n.r.	↑
Lock *et al.* (2005b)*	c9, t11/VA-enriched butter	↓	↓	→

* CLA esterified into triacylglycerol
n.r. = not reported

Functional food implications of CLA for disease prevention in humans

Cancer applications

Cancer takes many years to develop so documenting that functional food components are beneficial in health maintenance and the prevention of this disease is a major challenge. We are particularly interested in breast cancer; because CLA could be a unique chemo-preventive agent due to its favoured accumulation in breast tissue adipocytes as discussed earlier. Breast cancer is the most commonly diagnosed cancer in women and the leading cause of cancer mortality in females in the world (Parkin, 2001). However, other than the mutation of the BRCA genes, little is known about the etiologic factors for breast cancer (Ip *et al.*, 2003). Thus, knowledge is sufficiently limited that it is not possible to institute primary intervention strategies to remove causative agents from the environment. Nevertheless, the milestone tamoxifen trial convincingly demonstrated that breast cancer prevention in high-risk women can be successful (Fisher *et al.*, 1998). Results from this trial provide a strong rational for developing effective preventative strategies and the use of a functional food approach would have many advantages as a preventative strategy.

Assessing the functional food role of CLA in the prevention of cancer, including breast cancer, presents some unusual difficulties. The use of cancer morbidity as an endpoint in prevention trials is a costly, long-term endeavor and estimating or maintaining differences in the dietary intake of a functional food component for a disease with such a very long latency period is questionable. The application processes would be hastened by using biomarkers as potential surrogate endpoints

to predict reduced cancer risk, but to date there are no consensus biomarkers for most types of cancer, including breast cancer. In addition, simply knowing the intake of dairy products is not sufficient; CLA is associated with the fat and dairy products vary widely in fat content. Even when the CLA content is expressed on a fat basis, a several-fold variation in CLA within a dairy product line is still observed (Riel, 1963; Kelly and Bauman, 1996; Jensen, 2002) due to the effects of dietary practices and individual cow differences in dairy production as discussed earlier. Furthermore, butter (i.e. CLA) is often added to other foods during manufacturing and is an ingredient in many recipes (Subar *et al.*, 1998). Endogenous synthesis is also a factor and the kinetic study by Turpeinen *et al.* (2002) demonstrated a substantial variation among individuals in the extent of VA conversion to CLA. Finally, the adequacy of the analysis of CLA isomers in the existing databases for fatty acid composition of foods is a major limitation; an example of this is the fatty acid composition of foods reported for the recent TRANSFAIR study as discussed by Wolff and Precht (2002).

The above discussion clearly demonstrates that evaluating the specific role of CLA in health maintenance and the prevention of cancer will be very difficult, and many of the approaches used will have substantial limitations. Nevertheless, several epidemiological studies have been reported. An initial case-control study with Finnish women found an inverse association between dietary intake and serum concentrations of CLA and breast cancer in postmenopausal women, but not in premenopausal women (Aro *et al.*, 2000). After adjusting for known risk factors, postmenopausal women in the highest quintile of CLA had a 3- to 5-fold lower risk of breast cancer. Since then two other epidemiological studies have failed to confirm a protective role for CLA in breast cancer risk. A Dutch cohort study used dietary assessment based on a food-frequency questionnaire and the fatty acid analysis of foods reported in the TRANSFAIR study (Voorrips *et al.*, 2002) and a French study comparing CLA content in tissue adjacent to malignant tumors with levels in tissue adjacent to benign tumors (Chajès *et al.*, 2002). A recent US study by McCann *et al.* (2004) examined breast cancer risk and dietary intake of *cis*-9, *trans*-11 CLA in women that had histologically confirmed breast cancer or were matched controls. They found that there were nonsignificant inverse associations between intake of *cis*-9, *trans*-11 CLA and incidence of breast cancer in premenopausal but not postmenopausal women. The benefit was more apparent in women with the more aggressive estrogen receptor negative tumors. These studies faced some of the difficulties cited above and in addition the Dutch and French studies have methodological inadequacies that have been discussed elsewhere (Wolff and Precht, 2002; Parodi, 2004).

Atherosclerosis applications

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries (Mangiapane and Salter, 1999). It is the primary cause of heart disease and stroke in the Western world, being responsible for 50% of all deaths. A number of genetic and environmental risk factors have been identified with the relative abundance of the different lipoproteins being of primary importance as discussed earlier (Lusis, 2000). To date, there have been no epidemiological studies that specifically compared the intake of CLA derived from functional foods with the risk of CHD, although such studies would face many of the difficulties discussed previously for evaluating the role of CLA in the prevention of cancer,

including accurate assessment of dietary levels. However, several studies have examined the effects of dietary supplements of CLA on plasma lipid variables. These investigations have been mainly focused on the anti-obesity effects of CLA and blood lipids were of secondary interest. Supplements have been generally composed of a mixture of CLA isomers and effects on blood lipids and lipoproteins were equivocal as reviewed by Gaullier *et al.* (2002) and O'Shea (2005).

The functional food role for CLA in dairy products relates to the *cis*-9, *trans*-11 CLA isomer, whereas the above studies on obesity and body composition are related to effects of the *trans*-10, *cis*-12 isomer. Recently, there have been two studies that provide insight on *cis*-9, *trans*-11 CLA and both focused on blood lipids in healthy subjects. Noone *et al.* (2002) demonstrated that a CLA supplement containing a 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers significantly improved plasma triacylglycerol and VLDL metabolism. Importantly, an 80:20 CLA isomer blend (*cis*-9, *trans*-11 CLA:*trans*-10, *cis*-12 CLA) significantly reduced VLDL-cholesterol concentrations, providing further evidence for the role of *cis*-9, *trans*-11 CLA in altering hepatic lipid metabolism. In support, Tricon *et al.* (2004) have reported the first investigation to utilize relatively pure CLA isomers and they observed that *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA had opposing effects on blood lipids in healthy humans. Plasma triacylglycerol, total plasma cholesterol, LDL-cholesterol and LDL:HDL-cholesterol were all lower during supplementation with *cis*-9, *trans*-11 CLA as compared to *trans*-10, *cis*-12 CLA (Tricon *et al*., 2004). This same group has also shown that both CLA isomers are readily incorporated in plasma and cellular lipids to a similar extent and in a dose-dependent manner (Burdge *et al*., 2004). Thus, while data are limited, these two studies provide support that some of the cardio-protective effects of CLA reported in animal models will extend to humans.

Consideration of atherosclerosis and *trans* fatty acids

Cis-9, *trans*-11 CLA and VA are both *trans* fatty acids (TFA) and current public health policy strongly recommends a reduction in the intake of TFA based on the possible adverse effects related to their putative association with elevated plasma concentrations of cholesterol and LDL along with lower concentrations of HDL (Institute of Medicine, 2002; Williams, 2000). A number of epidemiological studies have investigated the relationship between dietary intake of TFA and risk of CHD, and these are cited as strong evidence for the need to reduce the intake of TFA (Institute of Medicine, 2002). The intake of TFA used in these evaluations would predominantly include *trans* 18:1 isomers, and little or no distinction has been made between the biological effects of different *trans* isomers. Most of the analyses relate to TFA from partially hydrogenated vegetable oils (PHVO) and results have been broadly extrapolated to imply that high consumption of any and all *trans* fatty acid isomers is associated with increased CHD. However, the position of the double bond in fatty acids is an important consideration in terms of biological effects and as a consequence isomeric differences between food sources of TFA need to be considered.

Some epidemiological and cohort studies have data that allow a comparison of TFA intake from different food sources and results indicate the positive association with CHD risk can be explained entirely by the intake of TFA from vegetable sources (Ascherio *et al*., 1993; Bolton Smith *et al*., 1996; Gillman *et al*., 1997; Pietinen *et al*., 1997; Willett *et al*., 1993). In fact, in three of these studies (Bolton Smith *et al*., 1996; Pietinen *et al*., 1997; Willett *et al*., 1993) there was a negative association

between the intake of TFA of animal origin and the risk of CHD. For example, the Nurses' Health Study (Willett *et al*., 1993) reported that as the intake of TFA from vegetable fats progressively increased, the relative risk of CHD also increased with a risk of 1.78 at the highest quintile (Table 6). In contrast, CHD risk decreased with increasing intake of TFA from animal sources. Similarly, Gillman *et al.* (1997) showed a rise in incidence of CHD with increasing margarine intake, but not with increasing intake of butter. Case-control studies from Greece (Tzonou *et al*., 1993) and Italy (Tavani *et al*., 1997) also indicated that medium or high intake of margarine was associated with increased risk of CHD. During the period when these studies were conducted, margarines would have contained substantial quantities of TFA (Parodi, 2004). It has also been reported that the intake of *trans*-9 and *trans*-10 18:1 were positively correlated with CHD, whereas the intake of VA was not (Hodgson *et al*., 1996). Furthermore, a German case-control study reported that subjects with angiographically documented CHD had less *cis*-9, *trans*-11 CLA in their adipose tissue (i.e. less intake of ruminant fats) than control subjects (Fritsche *et al*., 1998). One exception was a study by Meijer *et al*. (2001) in hamsters, where there were no differences in the effects of *trans*-9 18:1 and VA on blood lipoprotein concentrations. Overall, results are very consistent that TFA from animal sources do not impose an increased risk of CHD and based on this it has been proposed that evolution and long-term coexistence have adapted humans to cope with the type of TFA present in foods derived from ruminants (Ackman, 1997).

Based on the above findings, we propose that TFA from ruminant fats differ in their relationship to the risk of CHD, and we suggest that this relates to differences in the specific fatty acid isomers in the fat sources. The *trans*-9 and *trans*-10 18:1 isomers predominate in PHVO whereas VA is the predominant isomer in ruminant fat, which also contains considerable quantities of CLA. We consider the most significant difference between VA and other *trans* 18:1 isomers is that VA can be converted to *cis*-9, *trans*-11 CLA via the enzyme Δ^9-desaturase. As mentioned previously, several studies have established that humans are capable of this conversion (Palmquist *et al*., 2005). Approximately 20% of VA is converted to CLA in humans (Turpeinen *et al*., 2002), thereby doubling CLA supply. Thus, there is an urgent need for further research into the effects of different *trans* isomers on human health. This is particularly important, as all current strategies for enriching ruminant products with CLA result in significant increases in the content of VA. For these products to be accepted, consumers must be confident that the benefits of increased CLA intake are not offset by detrimental effects of increased consumption of *trans* fatty acids.

Table 6. Epidemiological investigation of the relative risk of coronary heart disease in the Nurses' Health Study.[1]

	Relative risk in Quintile				
***Trans* isomer source**	**1**	**2**	**3**	**4**	**5**
Vegetable fats	1.00	1.43	1.11	1.39	1.78
Animal fats	1.00	0.76	0.69	0.55	0.59

[1]Study involved 69,181 women. Adapted from Willet *et al*. (1993).

CLA intake for health maintenance

Biomedical studies with animal models clearly establish that CLA is a promising candidate as a functional food component with the strongest data coming from its potential role as a natural anticarcinogen for the prevention of cancer, particularly breast cancer. However, the amount of *cis*-9, *trans*-11 CLA providing a protective effect in humans remains speculative. It is important to keep in mind that CLA is only one of several naturally occurring anticarcinogens that have been identified in dairy products and other foods (National Research Council, 1996; Parodi, 2004), and thus human diets would include several sources of anticarcinogens whereas our animal model studies are designed to focus on a single anticarcinogen. Additionally, in biomedical studies with animal models, massive doses of carcinogens are given so that all experimental animals develop numerous tumors over a short period of time; thus, an equally large dose of anticarcinogen is needed to have biological effects in these models. Nevertheless, the dietary intake of CLA to obtain a beneficial effect on health maintenance merits some consideration, even if it is highly speculative.

An early estimate of the level that might prove beneficial was 3 g/d of CLA for a 70 kg person; this represented a direct extrapolation based on body weight from the CLA dose of 0.1% of the diet found effective in reducing chemically-induced mammary tumors in rats (350 g body weight; Ip *et al*., 1994). However, as we learned more about the biology of CLA an extrapolation based on metabolic rate would seem more appropriate (Terpstra, 2001) and this basis for extrapolation gives an estimate of 700 to 800 mg/d of CLA required for humans (Watkins and Li, 2003; Parrish *et al*., 2003). As discussed in an earlier section, the average dietary intake of CLA is in the range of 100 to 300 mg/d, but this does not account for endogenous synthesis of CLA. Humans have an active Δ^9-desaturase that allows dietary VA to be converted to CLA (Adlof *et al*., 2000; Salminen *et al*., 1998), and tracer studies indicate that humans convert about 20% of the dietary VA to *cis*-9, *trans*-11 CLA (Turpeinen *et al*., 2002). Based on the relationship between VA:*cis*-9, *trans*-11 CLA typically observed for ruminant fat, Parodi (2003) suggested the CLA intake from ruminant–derived foods be multiplied by 1.4 to obtain an estimate of the "effective" CLA intake. Applying this, the effective CLA intake is currently in the range of 140 to 420 mg/d which is within 2- to 5-fold of the CLA intake estimated to offer protective value. Clearly, the protective benefits of dietary CLA intake are achievable using functional food products that have been naturally enhanced in VA and CLA. It is of special importance to achieve these CLA intakes using enriched products rather than simply increasing the consumption of standard products so that the intake of saturated fat from these foods will not be increased and may even be reduced.

Synthetic preparations of CLA are available as diet supplements, so this could represent an alternative source to functional foods. However, sources differ in the profile of CLA isomers and this may be important. Although some of the synthetic sources of CLA are of dubious quality (Yurawecz *et al*., 1999), for the most part they have equal portions of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers as major CLA components. Recent investigations have reported that CLA supplements cause some undesirable effects, and these have been specifically attributable to *trans*-10, *cis*-12 CLA. These potential health risks include the development of hyperinsulinemia and insulin resistance, and have been occasionally observed in studies with animal models and obese humans with metabolic syndrome (see reviews by Kelley and Erickson, 2003; Taylor

and Zahradka, 2004). However, in humans this has not been observed in healthy subjects or when isomer mixtures are used (Smedman and Vessby, 2001; Noone *et al*., 2002; Tricon *et al*., 2004). There have been no similar observations of hyperinsulinemia and insulin resistance in studies using supplements of *cis*-9, *trans*-11 CLA, but the production of capsules containing only this CLA isomer may not be economically viable. On the other hand, *cis*-9, *trans*-11 CLA is the predominant isomer in dairy products and VA/CLA enriched functional foods could be a viable way of delivering this unique CLA isomer to aid in the health maintenance of the general public.

CLA and consideration of saturated fats

For half a century, the concept of eating healthy has become synonymous with avoiding dietary fat, especially saturated fat (Katan *et al*., 1995; Morrison *et al*., 1950). This is important in our consideration of functional food approaches involving CLA and VA because both are fatty acids in milk fat, and milk fat is considered rich in saturated fat. The recommendation to specifically reduce dietary intake of saturated fatty acids principally arises from epidemiological evidence that raised plasma cholesterol represents a primary, independent risk factor for the development of premature atherosclerosis, e.g the Framingham Study (Dawber, 1980) and the Multiple Risk Factor Intervention Trial (MRFIT; Stamler *et al*., 1986) coupled with experimental evidence that raised plasma cholesterol is associated with high levels of saturated fat consumption (Keys *et al*., 1965; Hegsted *et al*., 1965). HMGCoA reductase inhibitors (cholesterol-lowering drugs) clearly reduce cardiovascular morbidity and mortality (Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al*., 1995), but at least part of this effect may be as a result of changes in oxidative stress and inflammatory processes within the artery wall (Koh *et al*., 2004). Despite these observations it has proven more difficult to establish that reducing plasma cholesterol through altering saturated fat intake has direct effects on cardiovascular morbidity and mortality (Hooper *et al*., 2001; Ravnskov, 1998). Some recent reviews of epidemiologic and experimental studies examining the relationships among dietary cholesterol, fats, and high serum cholesterol with atherosclerosis and cardiovascular disease have concluded that these links are inconclusive and results are often contradictory (German and Dillard, 2004; Hu *et al*., 1999; Parodi, 2004; Ravnskov, 1998; Ravnskov, 2002). In reviewing the history and politics behind the diet-heart hypothesis, Taubes (2001) concluded that after 50 years of research, there was little evidence that a diet low in saturated fat prolongs life. Clearly, the relationship of fats and cholesterol to CHD is more complex than initially thought and the risk of cardiovascular disease is multifaceted (Mangiapane and Salter, 1999).

The Nutrition Committee of the American Heart Association has emphasized the diversity in the biological effects of individual fatty acids and the need to evaluate specific fatty acids with respect to a range of variables related to the risk of CHD (Kris-Etherton *et al*., 2001). About 60% of the fatty acids in milk fat are saturated (Table 2) and fatty acids differ in their effects on blood cholesterol. In particular, lauric (12:0), myristic (14:0) and palmitic (16:0) acids, can increase blood levels of total cholesterol and LDL-cholesterol when added as dietary supplements (Kris-Etherton and Yu, 1997; Nicolosi, 1997), but these represent only about 40% of the saturated fatty acids in milk fat and their effects may be, at least partly, offset by a concomitant increase in the levels of antiatherogenic HDL cholesterol (Mensink *et al*., 2003). However, individuals do not

consume saturated fatty acids as a dietary entity, but rather as fats in food. Milk fat intake has been shown to be negatively associated with serum cholesterol in children (Samuelson *et al*., 2001) and a range of cardiovascular risk factors in older adults (Smedman *et al*., 1999; Warensjo *et al*., 2004). Furthermore, as reviewed by Parodi (2004), a number of epidemiological studies have found no association or a slight beneficial association between intake of milk and dairy products with variables related to the risk of CHD (e.g. Elwood *et al*., 2004; Gartside *et al*., 1998; Hu *et al*., 1999). Overall, the evidence that consumption of dairy products adversely affects the risk of CHD is equivocal. Nevertheless, by consuming dairy products with an enhanced content of CLA/VA (Table 2), higher levels of these beneficial fatty acids can be obtained without increasing the intake of saturated fat.

Conclusion

Research in animal science has traditionally focused on the productivity and well-being of food-producing animals. However, there is also a growing recognition of the consumer desire for more healthy and nutritious foods. The contributions of animal-derived foods in supplying essential nutrients have been recognized for some time, but consumers are increasingly aware that foods also contain components that can have positive effects on health maintenance and disease prevention. Referred to as "functional foods", conjugated linoleic acid (CLA) in milk fat is such a component. Milk fat contains many isomers of CLA, but *cis*-9, *trans*-11 CLA predominates and is the isomer with functional food potential in relation to cancer and atherosclerosis. The uniqueness of CLA in ruminant-derived foods is related to rumen biohydrogenation of unsaturated fatty acids. Research over the last decade has established that CLA in milk fat originates mainly from endogenous synthesis by mammary Δ^9-desaturase from vaccenic acid (VA), a biohydrogenation intermediate produced in the rumen. Thus, an understanding of dietary lipid supply, rumen fermentation and mammary synthesis of fat is required, and strategies to increase milk fat CLA center on enhancing rumen output of VA and increasing tissue activity of Δ^9-desaturase.

The anticarcinogenic activity of CLA has been consistently demonstrated with animal models and *in vitro* studies for a wide range of cancer types. The anticancer effects of CLA have been particularly impressive in biomedical models of breast cancer. By comparison, investigations of CLA effects on atherosclerosis are more limited. A number of studies using animal models have reported that dietary supplementation with mixtures of CLA isomers can improve the plasma profile of lipids and lipoproteins, reduce the development of atherosclerotic lesions, and indeed even induce the regression of pre-existing lesions. The CLA used for most studies of cancer and atherosclerosis has been a mixture of isomers synthetically produced from vegetable oils. Of special importance are recent results showing that beneficial effects were also achieved when the dietary supply of CLA was provided by a natural food. These studies are among the first to demonstrate the feasibility of a functional food approach using the natural form of CLA (esterified *cis*-9, *trans*-11 isomer) in a naturally-enriched food (dairy products). Furthermore, these investigations also established that the VA in milk fat is a functional food component with beneficial effects on cancer and atherosclerosis because it can be used for endogenous synthesis of CLA in humans.

Extending the results from biomedical models to implications of CLA and VA as functional food components in humans is limited. These evaluations are challenging and problematic because the development of cancer and atherosclerosis has long latency periods and there is a lack of consensus biomarkers, especially for cancer. Further, there have been significant advances in the analysis of CLA and specific *trans* fatty acids, and these techniques have not yet been applied to update values for food databases. Finally, successful application of functional food components found in milk fat includes the education of the public that not all fatty acids are equal. This is of special importance with the introduction of food labeling of *trans* fatty acid content as undesirable and the fact that both VA and *cis*-9, *trans*-11 CLA are *trans* fatty acids. Overall, studies summarized in this review demonstrate the exciting potential of CLA/VA enriched dairy products as functional foods that may provide benefits in health maintenance and disease prevention as well as improve the public perception of milk.

References

AbuGhazaleh, A.A. & T.C. Jenkins (2004). Short communication: Docosahexaenoic acid promotes vaccenic acid accumulation in mixed ruminal cultures when incubated with linoleic acid. Journal of Dairy Science, 87, 1047-1050.

AbuGhazaleh, A.A., D.J. Schingoethe, A.R. Hippen & K.F. Kalscheur (2003). Milk conjugated linoleic acid response to fish oil supplementation of diets differing in fatty acid profiles. Journal of Dairy Science, 86, 944-953.

Ackman, R.G. (1997). Has evolution and long-term coexistence adapted us to cope with *trans* fatty acids? Journal of Food Lipids, 4, 295-318.

Adlof, R.O., S. Duval & E.A. Emken (2000). Biosynthesis of conjugated linoleic acid in humans. Lipids, 35, 131-135.

Aimutis, W.R. (2004). Bioactive properties of milk protein with particular focus on anticariogenesis. Journal of Nutrition, 134, 989S-995S.

Aro, A., S. Männistö, L. Salminen, M.-L. Ovaskainen, V. Kataja & M. Uusitupa (2000). Inverse association between dietary and serum conjugated linoleic acid and risk of breast cancer in postmenopausal women. Nutrition and Cancer, 38, 151-157.

Ascherio, A., C. Hennekens, J. Buring, C. Master, M. Stampfer & W.C. Willet (1993). A case-control study of trans-fatty-acids intake and risk of myocardial-infarction. Circulation, 87, 702.

Auldist, M.J., J.K. Kay, N.A. Thomson, A.R. Napper & E.S. Kolver (2002). Brief communication: Concentrations of conjugated linoleic acid in milk from cows grazing pasture or fed a total mixed ration for an entire lactation. Proceedings of the New Zealand Society for Animal Production, 62, 240-247.

Baer, R.J., J. Ryali, D.J. Schingoethe, K.M. Kasperson, D.C. Donovan, A.R. Hippen & S.T. Franklin (2001). Composition and properties of milk and butter from cows fed fish oil. Journal of Dairy Science, 84, 345-353.

Banni, S., E. Angioni, E. Murru, G. Carta, M.P. Melis, D.E. Bauman, Y. Dong & C. Ip (2001). Vaccenic acid feeding increases tissue levels of conjugated linoleic acid and suppresses development of premalignant lesions in rat mammary gland. Nutrition and Cancer, 41, 91-97.

Banni, S., G. Carta, M.S. Contini, E. Angioni, M. Deiana, M.A. Dessi, M.P. Melis & F.P. Corongiu (1996). Characterization of conjugated diene fatty acids in milk, dairy products and lamb tissues. Nutritional Biochemistry, 7, 150-155.

Banni, S., S.D. Heys & K.W.J. Wahle (2003). Conjugated linoleic acids as anticancer nutrients: Studies *in vivo* and cellular mechanisms. In: J.-L. Sébédio, W.W. Christie & R.O. Adlof (eds.) Advances in Conjugated Linoleic Acid Research. Volume 2, pp. 267-282, AOCS Press, Champaign, IL.

Bauman, D.E., D.M. Barbano, D.A. Dwyer & J.M. Griinari (2000). Technical note: Production of butter with enhanced conjugated linoleic acid for use in biomedical studies with animal models. Journal of Dairy Science, 83, 2422-2425.

Bauman, D.E., L.H. Baumgard, B.A. Corl & J.M. Griinari (2001). Conjugated linoleic acid (CLA) and the dairy cow. In: P.C. Garnsworthy & J. Wiseman (eds.) Recent Advances in Animal Nutrition 2001. pp. 221-250, Nottingham University Press, Nottingham, UK.

Bauman, D.E., B.A. Corl & D.G. Peterson (2003). The biology of conjugated linoleic acids in ruminants. In: J.-L. Sébédio, W.W. Christie & R.O. Adlof (eds.) Advances in Conjugated Linoleic Acid Research. Volume 2, pp. 146-173, AOCS Press, Champaign, IL.

Bauman, D.E. & J.M. Griinari (2001). Regulation and nutritional manipulation of milk fat: low-fat milk syndrome. Livestock Production Science, 70, 15-29.

Bauman, D.E. & J.M. Griinari (2003). Nutritional regulation of milk fat synthesis. Annual Review of Nutrition, 23, 203-227.

Bauman, D.E., J.W. Perfield & A.L. Lock (2004). Effect of *trans* fatty acids on milk fat and their impact on human health. Proceedings of the Southwest Nutrition Conference, Tempe, AZ. pp. 41-52.

Belury, M.A. (2002). Dietary conjugated linoleic acid in health: physiological effects and mechanisms of action. Annual Review of Nutrition, 22, 505-531.

Belury, M.A., S.Y. Moya-Camarena, M. Lu, L.L. Shi, L.M. Leesnitzer & S.G. Blanchard (2002). Conjugated linoleic acid is an activator and ligand for peroxisome proliferator-activated receptor-gamma (PPAR gamma). Nutrition Research, 22, 817-824.

Bessa, R.J.B., J. Santos-Silva, J.M.R. Ribeiro & A.V. Portugal (2000). Reticulo-rumen biohydrogenation and the enrichment of ruminant edible products with linoleic acid conjugated isomers. Livestock Production Science, 63, 201-211.

Bickerstaffe, R. & A.R. Johnson (1972). The effect of intravenous infusions of sterculic acid on milk fat synthesis. British Journal of Nutrition, 27, 561-570.

Billett, M.A., J.S. Bruce, D.A. White, A.J. Bennett & A.M. Salter (2000). Interactive effects of dietary cholesterol and different saturated fatty acids on lipoprotein metabolism in the hamster. British Journal of Nutrition, 84, 439-447.

Bolton Smith, C., M. Woodward, S. Fenton & C.A. Brown (1996). Does dietary *trans* fatty acid intake relate to the prevalence of coronary heart disease in Scotland? European Heart Journal, 17, 837-845.

Booth, R.G., W.J. Dann, S.K. Kon & T. Moore (1933). A new variable factor in butter fat. Chemistry and Industry, 52, 270.

Burdge, G.C., B. Lupoli, J.J. Russell, S. Tricon, S. Kew, T. Banerjee, K.J. Shingfield, D.E. Beever, R.F. Grimble, C.M. Williams, P. Yaqoob & P.C. Calder (2004). Incorporation of *cis*-9, *trans*-11 or *trans*-10, *cis*-12 conjugated linoleic acid into plasma and cellular lipids in healthy men. Journal of Lipid Research, 45, 736-741.

Chajès, V., F. Lavillonnière, P. Ferrari, M.-L. Jourdan, M. Pinault, V. Maillard, J.-L. Sébédio & P. Bougnoux (2002). Conjugated linoleic acid content in breast adipose tissue is not associated with the relative risk of breast cancer in a population of French patients. Cancer Epidemiology, Biomarkers and Prevention, 11, 672-673.

Chilliard, Y., A. Ferlay & M. Doreau (2001). Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. Livestock Production Science, 70, 31-48.

Chilliard, Y., A. Ferlay, R.M. Mansbridge & M. Doreau (2000). Ruminant milk fat plasticity: Nutritional control of saturated, polyunsaturated, *trans* and conjugated fatty acids. Annual Zootechnology, 49, 181-205.

Chouinard, P.Y., L. Corneau, W.R. Butler, Y. Chilliard, J.K. Drackley & D.E. Bauman (2001). Effect of dietary lipid source on conjugated linoleic acid concentrations in milk fat. Journal of Dairy Science, 84, 680-690.

Corl, B.A., D.M. Barbano, D.E. Bauman & C. Ip (2003). *cis*-9, *trans*-11 CLA derived endogenously from *trans*-11 18:1 reduces cancer risk in rats. Journal of Nutrition, 133, 2893-2900.

Corl, B.A., L.H. Baumgard, D.A. Dwyer, J.M. Griinari, B.S. Phillips & D.E. Bauman (2001). The role of Δ^9-desaturase in the production of *cis*-9, *trans*-11 CLA. Journal of Nutritional Biochemistry, 12, 622-630.

Dawber, T.R. (1980). The Framingham Study: The Epidemiology of Atherosclerotic Disease. Harvard University Press, Cambridge, MA.

Dawson, R.M.C., N. Hemington & G.P. Hazlewood (1977). On the role of higher plant and microbial lipases in the ruminal hydrolysis of grass lipids. British Journal of Nutrition, 38, 225-232.

Dawson, R.M.C. & P. Kemp (1970). Biohydrogenation of dietary fats in ruminants. In: A.T. Phillipson (ed.) Physiology of Digestion and Metabolism in the Ruminant. pp. 504-518, Oriel Press, Newcastle upon Tyne, UK.

de Deckere, E.A.M., J.M.M. van Amelsvoort, G.P. McNeill & P. Jones (1999). Effects of conjugated linoleic acid (CLA) isomers on lipid levels and peroxisome proliferation in the hamster. British Journal of Nutrition, 82, 309-317.

Dhiman, T.R., G.R. Anand, L.D. Satter & M.W. Pariza (1999). Conjugated linoleic acid content of milk from cows fed different diets. Journal of Dairy Science, 82, 2146-2156.

Dhiman, T.R., L.D. Satter, M.W. Pariza, M.P. Galli, K. Albright & M.X. Tolosa (2000). Conjugated linoleic acid (CLA) content of milk from cows offered diets rich in linoleic and linolenic acid. Journal of Dairy Science, 83, 1016-1027.

Elwood, P.C., J.E. Pickering, J. Hughes, A.M. Fehily & A. Ness (2004). Milk drinking, ischaemic heart disease and ischaemic stroke 11. Evidence from cohort studies. European Journal of Clinical Nutrition, 58, 718-724.

Fisher, B., J.P. Costantino, D.L. Wickerham, C.K. Redmond, M. Kavanah, W.M. Cronin, V. Vogel, A. Robidoux, N. Dimitrov, J. Atkins, M. Daly, S. Wieand, E. Tan-Chiu, L. Ford, N. Wolmark & other National Surgical Adjuvant Breast and Bowel Project Investigators (1998). Tamoxifen for prevention of breast cancer: Report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. Journal of the National Cancer Institute, 90, 1371-1388.

Fritsche, J., H. Steinhart, V. Kardalinos & G. Klose (1998). Contents of *trans* fatty acids in human substernal adipose tissue and plasma lipids: Relation to angiographically documented coronary heart disease. European Journal of Medical Research, 3, 401-406.

Gartside, P.S., P.Wang & C.J. Glueck (1998). Prospective assessment of coronary heart disease risk factors: the NHANES 1 epidemiologic follow up study (NHEFS) 16-year follow-up. Journal of American College of Nutrition, 17, 263-269.

Gaullier, J.M., G. Berven, H. Blankson & O. Gudmundsen (2002). Clinical trial results support a preference for using CLA preparations enriched with two isomers rather than four isomers in human studies. Lipids, 37, 1019-1025.

Gavino, V.C., G. Gavino, M.J. Leblanc & B. Tuchweber (2000). An isomeric mixture of conjugated linoleic acids but not pure *cis*-9, *trans*-11-octadecadienoic acid affects body weight gain and plasma lipids in hamsters. Journal of Nutrition, 130, 27-29.

German, J.B. & C.J. Dillard (2004). Saturated fats: what dietary intake? American Journal of Clinical Nutrition, 80, 550-559.

Gill, H.S. & M.L. Cross (2000). Anticancer properties of bovine milk. British Journal of Nutrition, 84, S161-S166.

Gillman, M.W., L.A. Cupples, D. Gagnon, B.E. Millen, R.C. Ellison & W.P. Castelli (1997). Margarine intake and subsequent coronary heart disease in men. Epidemiology, 8, 144-149.

Griinari, J.M. & D.E. Bauman (1999). Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. In: M.P. Yurawecz, M.M. Mossoba, J.K.G. Kramer, M.W. Pariza & G. Nelson (eds.) Advances in Conjugated Linoleic Acid Research. Volume 1, pp. 180-200, AOCS Press, Champaign, IL.

Griinari, J.M., B.A. Corl, S.H. Lacy, P.Y. Chouinard, K.V.V. Nurmela & D.E. Bauman (2000). Conjugated linoleic acid is synthesized endogenously in lactating dairy cows by Δ^9-desaturase. Journal of Nutrition, 130, 2285-2291.

Harfoot, C.G. (1981). Lipid metabolism in the rumen. In: W.W. Christie (ed.) Lipid Metabolism in Ruminant Animals. pp. 21-55, Pergamon Press Ltd., Oxford, UK.

Harfoot, C.G. & G.P. Hazlewood (1997). Lipid metabolism in the rumen. In: P.N. Hobson & D.S. Stewart (eds.) The Rumen Microbial Ecosystem (Second Edition). pp. 382-426, Chapman & Hall, London, UK.

Harper, W.J. (2001). Biological properties of whey components. A review. The American Dairy Products Institute. Chicago, IL, 20pp.

Hegsted, D.M., R.B. McGandy, M.L. Myers & F.J. Stare (1965). Quantitative effects of dietary fat on serum cholesterol in man. American Journal of Clinical Nutrition, 17, 281-295.

Hodgson, J.M., M.L. Wahlqvist, J.A. Boxall & N.D. Balazs (1996). Platelet *trans* fatty acids in relation to angiographically assessed coronary artery disease. Atherosclerosis, 120, 147-154.

Hooper, L., C.D. Summerbell, J.P.T. Higgins, R.L. Thompson, N.E. Capps, G.D. Smith, R.A. Riemersma & S. Ebrahim (2001). Dietary fat intake and prevention of cardiovascular disease: Systematic review. British Medical Journal, 322, 757– 763.

Hu, F.B., M.J. Stampfer, J.E. Manson, A. Ascherio, G.A. Colditz, F.E. Speizer, C.H. Hennekens & W.C. Willett (1999). Dietary saturated fats and their food sources in relation to the risk of coronary heart disease in women. American Journal of Clinical Nutrition, 70, 1001-1008.

Institute of Medicine (1994). Opportunities in the Nutrition and Food Sciences: Research Challenges and the Next Generation of Investigators. National Academy of Science Press, Washington, D.C., 310pp.

Institute of Medicine (2002). Letter Report on Dietary Reference Intakes for *Trans* Fatty Acids. National Academy of Science Press, Washington, D.C., 23pp.

Ip, C., S. Banni, E. Angioni, G. Carta, J. McGinley, H.J. Thompson, D. Barbano & D.E. Bauman (1999). Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. Journal of Nutrition, 129, 2135-2142.

Ip, C., S.P. Briggs, A.D. Haegele, H.J. Thompson, J. Storkson & J.A. Scimeca (1996). The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet. Carcinogenesis, 17, 1045-1050.

Ip, C., S.F. Chin, J.A. Scimeca & M.A. Pariza (1991). Mammary cancer prevention by conjugated dieonic derivatives of linoleic acid. Cancer Research, 51, 6118-6124.

Ip, C., Y. Don, H.J. Thompson, D.E. Bauman & M.M. Ip (2001). Control of rat mammary epithelium proliferation by conjugated linoleic acid. Nutrition and Cancer, 39, 233-238.

Ip, C., J.A. Scimeca & H.J. Thompson (1995). Effect of timing and duration of dietary conjugated linoleic acid on mammary cancer prevention. Nutrition and Cancer, 24, 241-247.

Ip, C., M. Singh, H.J. Thompson & J.A. Scimeca (1994). Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Research, 54, 1212-1215.

Ip, M.M., P.A. Masso-Welch & C. Ip (2003). Prevention of mammary cancer with conjugated linoleic acid: role of the stroma and epithelium. Journal of Mammary Gland Biology and Neoplasia, 8, 103-118.

Jahreis, G., J. Fritsche, P. Mockel, F. Schone, U. Moller & H. Steinhart (1999). The potential anticarcinogenic conjugated linoleic acid, *cis*-9, *trans*-11 C18:2, in milk of different species: Cow, goat, ewe, sow, mare, woman. Nutritional Research, 19, 1541-1549.

Jeffcoat, R. & M.R. Pollard (1977). Studies on the inhibition of the desaturases by cyclopropenoid fatty acids. Lipids, 12, 480-485.

Jenkins, T.C. (1993). Lipid metabolism in the rumen. Journal of Dairy Science, 76, 3851-3863.

Jensen, R.G. (2002). Invited review: The composition of bovine milk lipids: January 1995 to December 2000. Journal of Dairy Science, 85, 295-350.

Katan, M.B., P.L. Zock & R.P. Mensink (1995). Dietary oils, serum lipoproteins, and coronary heart disease. American Journal of Clinical Nutrition, 61(Suppl.), 1368S-1373S.

Kay, J.K., T.R. Mackle, M.J. Auldist, N.A. Thomson & D.E. Bauman (2004). Endogenous synthesis of *cis*-9, *trans*-11 conjugated linoleic acid in dairy cows fed fresh pasture. Journal of Dairy Science, 87, 369-378.

Keeney, M. (1970). Lipid metabolism in the rumen. In: A.T. Phillipson (ed.) Physiology of Digestion and Metabolism in the Ruminant. pp. 489-503, Oriel Press, Newcastle upon Tyne, UK.

Kelley, D.S. & K.L. Erickson (2003). Modulation of body composition and immune cell functions by conjugated linoleic acid in humans and animal models: Benefits vs. risks. Lipids, 38, 377-386.

Kelly, M.L. & D.E. Bauman (1996). Conjugated linoleic acid: A potent anticarcinogen found in milk fat. Proceedings of the Cornell Nutrition Conference. pp. 68-74.

Kelly, M.L., J.R. Berry, D.A. Dwyer, J.M. Griinari, P.Y. Chouinard, M.E. Van Amburgh & D.E. Bauman (1998a). Dietary fatty acid sources affect conjugated linoleic acid concentrations in milk from lactating dairy cows. Journal of Nutrition, 128, 881-885.

Kelly, M.L., E.S. Kolver, D.E. Bauman, M.E. Van Amburgh & L.D. Muller (1998b). Effect of intake of pasture on concentrations of conjugated linoleic acid in milk of lactating cows. Journal of Dairy Science, 81, 1630-1636.

Kelsey, J.A., B.A. Corl, R.J. Collier & D.E. Bauman (2003). The effect of breed, parity, and stage of lactation on conjugated linoleic acid (CLA) in milk fat from dairy cows. Journal of Dairy Science, 86, 2588-2597.

Kemp, P. & D.J. Lander (1984). Hydrogenation *in vitro* of alpha-linolenic acid to stearic-acid by mixed cultures of pure strains of rumen bacteria. Journal of General Microbiology, 130, 527-533.

Kepler, C.R., K.P. Hirons, J.J. McNeill & S.B. Tove (1966). Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. Journal of Biological Chemistry, 241, 1350-1354.

Keys, A., J.T. Anderson & F. Grande (1965). Serum cholesterol response to changes in the diet. IV. Particular saturated fatty acids in the diet. Metabolism, 14, 776-787.

Koh, K.K., J.W. Son, J.Y. Ahn, D.K. Jin, H.S. Kim, Y.M. Choi, T.H. Ahn, D.S. Kim & E.K. Shin (2004). Vascular effects of diet and statin in hypercholesterolemic patients. International Journal of Cardiology, 95, 185-191.

Kraft, J., M. Collomb, P. Mockel, R. Sieber & G. Jahreis (2003). Differences in CLA isomer distribution of cow's milk lipids. Lipids, 38, 657-664.

Kramer, J.K.G., P.W. Parodi, R.G. Jensen, M.M. Mossoba, M.P. Yurawecz & R.O. Adlof (1998). Rumenic acid: A proposed common name for the major conjugated linoleic acid isomer found in natural products. Lipids, 33, 835.

Kris-Etherton, P., S.R. Daniels, R.H. Eckel, M. Engler, B.V. Howard, R.M. Krauss, A.H. Lichtenstein, F. Sacks, S. St. Jeor & M. Stampfer (2001). AHA scientific statement: Summary of the scientific conference on dietary fatty acids and cardiovascular health. Conference summary from the nutritional committee of the American Heart Association. Journal of Nutrition, 131, 1322-1326.

Kris-Etherton, P.M. & S. Yu (1997). Individual fatty acid effects on plasma lipids and lipoproteins: Human studies. American Journal of Clinical Nutrition, 65 (Suppl.), 1628S-1644S.

Kritchevsky, D. (2003). Conjugated linoleic acids in experimental atherosclerosis. In: J.-L. Sébédio, W.W. Christie & R.O. Adlof (eds.) Advances in Conjugated Linoleic Acid Research. Volume 2, pp. 293-301, AOCS Press, Champaign, IL.

Kritchevsky, D., S.A. Tepper, S. Wright & S.K. Czarnecki (2002). Influence of graded levels of conjugated linoleic acid (CLA) on experimental atherosclerosis in rabbits. Nutritional Research, 22, 1275-1279.

Kritchevsky, D., S.A. Tepper, S. Wright, S.K. Czarnecki, T.A. Wilson & R.J. Nicolsi (2004). Conjugated linoleic acid isomer effects in atherosclerosis: Growth and regression of lesions. Lipids, 39, 611-616.

Kritchevsky, D., S.A. Tepper, S. Wright, P. Tso & S.K. Czarnecki (2000). Influence of conjugated linoleic acid (CLA) on establishment and progression of atherosclerosis in rabbits. Journal of American College of Nutrition, 19, 472S-477S.

Lawless, F., J.J. Murphy, D. Harrington, R. Devery & C. Stanton (1998). Elevation of conjugated *cis*-9, *trans*-11-octadecadienoic acid in bovine milk because of dietary supplementation. Journal of Dairy Science, 81, 3259-3267.

Lawless, F., C. Stanton, P. L'Escop, R. Devery, P. Dillon & J.J. Murphy (1999). Influence of breed on bovine milk *cis*-9, *trans*-11-conjugated linoleic acid content. Livestock Production Science, 62, 43-49.

Lee, K.N., D. Kritchevsky & M.W. Pariza (1994). Conjugated linoleic acid and atherosclerosis in rabbits. Atherosclerosis, 108, 19-25.

Lock, A.L. & D.E. Bauman (2004). Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids, 39, 1197-1206.

Lock, A.L., D.E. Bauman & P.C. Garnsworthy (2005a). Effect of production variables on the *cis*-9, *trans*-11 conjugated linoleic acid content of cows' milk. Journal of Dairy Science, 88, 2714-2717.

Lock, A.L., B.A. Corl, D.M. Barbano, D.E. Bauman & C. Ip (2004). The anticarcinogenic effect of *trans*-11 18:1 is dependent on its conversion to *cis*-9, *trans*-11 CLA by Δ^9-desaturase in rats. Journal of Nutrition, 134, 2698-2704.

Lock, A.L. & P.C. Garnsworthy (2002). Independent effects of dietary linoleic and linolenic fatty acids on the conjugated linoleic acid content of cow's milk. Animal Science, 74, 163-176.

Lock, A.L. & P.C. Garnsworthy (2003). Seasonal variation in milk conjugated linoleic acid and Δ^9-desaturase activity in dairy cows. Livestock Production Science, 79, 47-59.

Lock, A.L., C.A.M. Horne, D.E. Bauman & A.M. Salter (2005b). Effect of butter enriched in conjugated linoleic acid (CLA) and vaccenic acid on plasma lipoproteins and tissue fatty acid composition in the cholesterol-fed hamster. Journal of Nutrition, 135, 1934-1939.

Lusis, A.J. (2000). Atherosclerosis. Nature, 407, 233-241.

Lynch, J.M., A.L. Lock, D.A. Dwyer, R. Noorbakhsh, D.M. Barbano & D.E. Bauman (2005). Flavor and stability of pasteurized milk with elevated levels of conjugated linoleic acid and vaccenic acid. Journal of Dairy Science, 88, 489-498.

Mangiapane, E.H. & A.M. Salter (1999). Diet, Lipoproteins and Coronary Heart Disease: A Biochemical Perspective. Nottingham University Press, Nottingham, UK, 180pp.

Masso-Welch, P.A., D. Zangani, C. Ip, M.M. Vaughan, S. Shoemaker, R.A. Ramirez & M.M. Ip (2002). Inhibition of angiogenesis by the cancer chemopreventive agent conjugated linoleic acid. Cancer Research, 62, 4383-4389.

McCann, S.E., C. Ip, M.M. Ip, M.K. McGuire, P. Muti, S.B. Edge, M. Trevisan & J.L. Freudenheim (2004). Dietary intake of conjugated linoleic acids and risk of premenopausal and postmenopausal breast cancer, Western New York exposures and breast cancer study (WEB Study). Cancer Epidemiology, Biomarkers and Prevention, 13, 1480-1484.

McLeod, R.S., A.M. LeBlanc, M.A. Langille, P.L. Mitchell & D.L. Currie (2004). Conjugated linoleic acids, atherosclerosis, and hepatic very low-density lipoprotein metabolism. American Journal of Clinical Nutrition, 79, 1169S-1174S.

Meijer, G.W., A. van Tol, T.J.C. van Berkel & J.A. Weststrate (2001). Effect of dietary elaidic versus vaccenic acid on blood and liver lipids in the hamster. Atherosclerosis, 157, 31-40.

Meir, K.S. & E. Leitersdorf (2004). Atherosclerosis in the apolipoprotein E-deficient mouse: A decade of progress. Arteriosclerosis, Thrombosis, and Vascular Biology, 24, 1-9.

Mensink, R.P., P.L. Zock, A.D.M. Kester & M.B. Katan (2003). Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: A meta-analysis of 60 controlled trials. American Journal of Clinical Nutrition, 77, 1146-1155.

Miller, G.D., J.K. Jarvis & L.D. McBean (2004). Handbook of Dairy Foods and Nutrition (Second Edition). CRC Press, New York, 423pp.

Milner, J.A. (1999). Functional foods and health promotion. Journal of Nutrition, 129, 1395S-1397S.

Moore, T. (1939). CCII. Spectroscopic changes in fatty acids. VI. General. Biochemical Journal, 33, 1635-1638.

Morrison, L.M., M. Awierlein & E. Wolfson (1950). The effects of low fat low cholesterol diets on the serum lipids. Circulation, 2, 475-476.

Munday, J.S., K.G. Thompson & K.A.C. James (1999) Dietary conjugated linoleic acids promote fatty streak formation in the C-57BL/6 mouse atherosclerosis model. British Journal of Nutrition, 81, 251-255.

National Research Council (1988). Designing Foods: Animal Product Options in the Marketplace. National Academy Press, Washington, D.C., 367pp.

National Research Council (1996). Carcinogens and Anticarcinogens in the Human Diet. National Academy Press, Washington, D.C., 417pp.

National Research Council (2003). Frontiers in Agricultural Research: Food, Health, Environment and Communities. National Academy Press, Washington, D.C., 239pp.

Navarro, V., A. Zabala, M.T. Macarulla, A. Fernandez-Quintela, V.M. Rodriguez, E. Simon & M.P. Portillo (2003). Effects of conjugated linoleic acid on body fat accumulation and serum lipids in hamsters fed an atherogenic diet. Journal of Physiology and Biochemistry, 59, 193-199.

Nicolosi, R.J. (1997). Dietary fat saturation effects on low-density-lipoprotein concentrations and metabolism in various animal models. American Journal of Clinical Nutrition, 65 (Suppl.), 1617S-1627S.

Nicolosi, R.J., E.J. Rogers, D. Kritchevsky, J.A. Scimeca & P.J. Huth (1997). Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. Artery, 22, 266-277.

Noone, E.J., H.M. Roche, A.P. Nugent & M.J. Gibney (2002). The effect of dietary supplementation using isomeric blends of conjugated linoleic acid on lipid metabolism in healthy human subjects. British Journal of Nutrition, 88, 243-251.

Offer, N.W., M. Marsden, J. Dixon, B.K. Speake & F.E. Thacker (1999). Effect of dietary fat supplements on levels of n-3 poly-unsaturated fatty acids, *trans* acids and conjugated linoleic acid in bovine milk. Animal Science, 69, 613-625.

O'Shea, M., M. Van Der Zee, and I. Mohede (2005). Conjugated linoleic acid sources and human studies. In: C.C. Akoh & O.M. Lai (eds.) Healthful Lipids. pp. 249-272, AOCS Press, Champaign, IL.

Palmquist, D.L. & T.C. Jenkins (1980). Fat in lactation rations: Review. Journal of Dairy Science, 63, 1-14.

Palmquist, D.L., A.L. Lock, K.J. Shingfield & D.E. Bauman (2005). Biosynthesis of conjugated linoleic acid in ruminants and humans. In: S.L. Taylor (ed.) Advances in Food and Nutrition Research. Volume 50, pp. 179-217, Elsevier Inc., San Diego, CA.

Pariza, M.W. (2004). Perspective on the safety and effectiveness of conjugated linoleic acid. American Journal of Clinical Nutrition, 79, 1132S-1136S.

Pariza, M.W., S.H. Ashoor, F.S. Chu & D.B. Lund (1979). Effects of temperature and time on mutagen formation in pan-fried hamburger. Cancer Letters, 7, 63-69.

Parkin, D.M. (2001). Global cancer statistics in the year 2000. Lancet Oncology, 2, 533-543.

Parodi, P.W. (1977). Conjugated octadecienoic acids of milk fat. Journal of Dairy Science, 60, 1550-1553.

Parodi, P.W. (1999). Conjugated linoleic acid and other anticarcinogenic agents of bovine milk fat. Journal of Dairy Science, 82, 1339-1349.

Parodi, P.W. (2002). Conjugated linoleic acid. In: H. Roginski, J.W. Furquay & P.F. Fox (eds.) Encyclopedia of Dairy Sciences. pp. 1587-1594, Elsevier Sciences Ltd., London, UK.

Parodi, P.W. (2003). Conjugated linoleic acid in food. In: J.-L. Sébébio, W.W. Christie & R.O. Adlof (eds.) Advances in Conjugated Linoleic Acid Research. Volume 2, pp. 101-122, AOCS Press, Champaign, IL.

Parodi, P.W. (2004). Milk fat in human nutrition. Australia Journal of Dairy Technology, 59, 3-59.

Parrish Jr., F.C., B.R. Wiegand, D.C. Beitz, D.U. Ahn, M. Du & A.H. Trenkle (2003). Use of dietary CLA to improve composition and quality of animal-derived foods. In: J.-L. Sébébio, W.W. Christie & R.O. Adlof (eds.) Advances in Conjugated Linoleic Acid Research. Volume 2, pp. 187-217, AOCS Press, Champaign, IL.

Peterson, D.G., J.A. Kelsey & D.E. Bauman (2002). Analysis of variation in *cis*-9, *trans*-11 conjugated linoleic acid (CLA) in milk fat of dairy cows. Journal of Dairy Science, 85, 2164-2172.

Phelps, R.A., F.S. Shenstone, A.R. Kemmerer & R.J. Evans (1965). A review of cyclopropenoid compounds: Biological effects of some derivatives. Poultry Science, 44, 358-394.

Pietinen, P., A. Ascherio, P. Korhonen, A.M. Hartman, W.C. Willett, D. Albanes & J. Virtamo (1997). Intake of fatty acids and risk of coronary heart disease in a cohort of Finnish men. American Journal of Epidemiology, 145, 876-887.

Piperova, L.S., J. Sampugna, B.B. Teter, K.F. Kalscheur, M.P. Yurawecz, Y. Ku, K.M. Morehouse & R.A. Erdman (2002). Duodenal and milk *trans* octadecenoic acid and conjugated linoleic acid (CLA) isomers indicate that postabsorptive synthesis is the predominant source of *cis*-9-containing CLA in lactating dairy cows. Journal of Nutrition, 132, 1235-1241.

Precht, D. & J. Molkentin (1999). C18:1, C18:2 and C18:3 *trans* and *cis* fatty acid isomers including conjugated *cis*-9, *trans*-11 linoleic acid (CLA) as well as total fat composition of German human milk lipids. Nahrung-Food, 43, 233-244.

Ramaswamy, N., R.J. Baer, D.J. Schingoethe, A.R. Hippen, K.M. Kasperson & L.A. Whitlock (2001b). Short communication: Consumer evaluation of milk high in conjugated linoleic acid. Journal of Dairy Science, 84, 1607-1609.

Ramaswamy, N., R.J. Baer, D.J. Schingoethe, A.R. Hippen, K.M. Kasperson & L.A. Whitlock (2001a). Composition and flavor of milk and butter from cows fed fish oil, extruded soybeans, or their combination. Journal of Dairy Science, 84, 2144-2151.

Ravnskov, U. (1998). The questionable role of saturated and polyunsaturated fatty acids in cardiovascular disease. Journal of Clinical Epidemiology, 51, 443 – 460.

Ravnskov, U. (2002). Hypothesis out-of-date: The diet-heart idea. Journal of Clinical Epidemiology, 55, 1057-1063.

Rickert, R., H. Steinhart, J. Fritsche, N. Sehat, M.P. Yurawecz, M.M. Mossoba, J.A.G. Roach, K. Eulitz, Y. Ku & J.K.G. Kramer (1999). Enhanced resolution of conjugated linoleic acid isomers by tandem-column silver-ion high performance liquid chromatography. Journal of High Resolution Chromatography, 22, 144-148.

Riel, R.R. (1963). Physico-chemical characteristics of Canadian milk fat: Unsaturated fatty acids. Journal of Dairy Science, 46, 102-106.

Ritzenthaler, K.L., M.K. McGuire, R. Falen, T.D. Shultz, N. Dasgupta & M.A. McGuire (2001). Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicate methodology. Journal of Nutrition, 131, 1548-1554.

Saebø, A. (2003). Commercial synthesis of conjugated linoleate. In: J.-L. Sébédio, W.W. Christie & R.O. Adlof (eds.) Advances in Conjugated Linoleic Acid Research. Volume 2, pp. 71-81, AOCS Press, Champaign, IL.

Salminen, I., M. Mutanen, M. Jauhiainen & A. Aro (1998). Dietary *trans* fatty acids increase conjugated linoleic acid levels in human serum. Journal of Nutritional Biochemistry, 9, 93-98.

Salter, A.M., E.H. Mangiapane, A.J. Bennett, J.S. Bruce, M.A. Billett, K.L. Anderton, C.B. Marenah, N. Lawson & D.A. White (1998). The effect of different fatty acids on lipoprotein metabolism: concentration-dependent effects of diets enriched in oleic, myristic, palmitic and stearic acids. British Journal of Nutrition, 79, 195-202.

Samuelson, G., L.-E. Bratteby, R. Mohsen & B. Vessby (2001). Dietary fat intake in healthy adolescents: inverse relationships between the estimated intake of saturated fatty acids and serum cholesterol. British Journal of Nutrition, 85, 333-341.

Scandinavian Simvastatin Survival Study Group (1994). Randomized trial of cholesterol lowering in 4444 patients with coronary heart disease: The Scandinavian Simvastatin Survival Study (4S). Lancet, 344, 1383-1389.

Scimeca, J.A. (1999). Cancer inhibition in animals. In: M.P. Yurawecz, M.M. Mossoba, J.K.G. Kramer, M.W. Pariza & G. Nelson (eds.) Advances in Conjugated Linoleic Acid Research. Volume 1, pp. 420-443, AOCS Press, Champaign, IL.

Shepherd, J., S. Cobbe, I. Ford, C. Isles, A. Lorimer, P. MacFarlane, J. McKillop & C. Packard (1995). Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. New England Journal of Medicine, 333, 1301-1307.

Sher, J., A. Pronczuk, T. Hajri & K.C. Hayes (2003). Dietary conjugated linoleic acid lowers plasma cholesterol during cholesterol supplementation, but accentuates the atherogenic lipid profile during the acute phase response in hamsters. Journal of Nutrition, 133, 456-460.

Shingfield, K.J., S. Ahvenjarvi, V. Toivonen, A. Arola, K.V.V. Nurmela, P. Huhtanen & J.M. Griinari (2003). Effect of dietary fish oil on biohydrogenation of fatty acids and milk fatty acid content in cows. Animal Science, 77, 165-179.

Smedman, A.E.M., I-B. Gustafsson, L.G.T. Berglund & B.O.H. Vessby (1999). Pentadecanoic acid in serum as a marker for milk fat: Relations between intake of milk fat and metabolic risk factors. American Journal of Clinical Nutrition, 69, 22-29.

Smedman, A. & B. Vessby (2001). Conjugated linoleic acid supplementation in humans – metabolic effects. Lipids, 36, 773-781.

Spady, D.K. & J.M. Dietschy (1983). Sterol synthesis *in vivo* in 18 tissues of the squirrel-monkey, guinea-pig, rabbit, hamster, and rat. Journal of Lipid Research, 24, 303-315.

Stamler, J., D. Wentworth & J.D. Neaton (1986). Is the relationship between serum cholesterol and risk of premature death from coronary heart disease continuous or graded? Journal of the American Medical Association, 256, 2823-2828.

Stanton, C., F. Lawless, G. Kjellmer, D. Harrington, R. Devery, J.F. Connolly & J. Murphy (1997). Dietary influences on bovine milk *cis*-9, *trans*-11-conjugated linoleic acid content. Journal of Food Science, 62, 1083-1086.

Stanton, C., J. Murphy, E. McGrath & R. Devery (2003). Animal feeding strategies for conjugated linoleic acid enrichment of milk. In: J.-L. Sébédio, W.W. Christie & R.O. Adlof (eds.) Advances in Conjugated Linoleic Acid Research. Volume 2, pp. 123-145, AOCS Press, Champaign, IL.

Subar, A.F., S.M. Krebs-Smith, A. Cook & L.L. Kahle (1998). Dietary sources of nutrients among US adults, 1989 to 1991. Journal of the American Dietetic Association, 98, 537-547.

Taubes, G. (2001). The soft science of dietary fat. Science, 291, 2535-2541.

Tavani, A., E. Negri, B. Davanzo & C. LaVecchia (1997). Margarine intake and risk of nonfatal acute myocardial infarction in Italian women. European Journal of Clinical Nutrition, 51, 30-32.

Taylor, C.G. & P. Zahradka (2004). Dietary conjugated linoleic acid and insulin sensitivity and resistance in rodent models. American Journal of Clinical Nutrition, 79(Suppl), 1164S-1168S.

Terpstra, A.H.M. (2001). Differences between humans and mice in efficacy of the body fat lowering effect of conjugated linoleic acid: Role of metabolic rate. Journal of Nutrition, 131, 2067-2068.

Thompson, H.J., Z.J. Zhu, S. Banni, K. Darcy, T. Loftus & C. Ip (1997). Morphological and biochemical status of the mammary gland as influenced by conjugated linoleic acid: Implication for a reduction in mammary cancer risk. Cancer Research, 57, 5067-5072.

Toomey, S., H. Roche, D. Fitzgerald & O. Belton (2003). Regression of pre-established atherosclerosis in the apoE(-/-) mouse by conjugated linoleic acid. Biochemistry Society Transactions, 31, 1075-1079.

Tricon, S., G.C. Burdge, S. Kew, T. Banerjee, J.I. Russell, E.J. Jones, R.F. Grimble, C.M. Williams, P. Yaqoob & P.C. Calder (2004). Opposing effects of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 conjugated linoleic acid on blood lipids in healthy humans. American Journal of Clinical Nutrition, 80, 614-620.

Turpeinen, A.M., M. Mutanen, A. Aro, I. Salminen, S. Basu, D.L. Palmquist & J.M. Griinari (2002). Bioconversion of vaccenic acid to conjugated linoleic acid in humans. American Journal of Clinical Nutrition, 76, 504-510.

Tzonou, A., A. Kalandidi, A. Trichopoulou, C.C. Hsieh, N. Toupadaki, W. Willett & D. Trichopoulos (1993). Diet and coronary heart disease - a case-control study in Athens, Greece. Epidemiology, 4, 511-516.

Urquhart, P., S.M. Parkin, J.S. Rogers, J.A. Bosley & A. Nicolaou (2002). The effect of conjugated linoleic acid on arachidonic acid metabolism and eicosanoids production in human saphenous vein endothelial cells. Biochimica Biophysica Acta, 1580, 150-160.

Valeille, K., D. Gripois, M.F. Bloquit, M. Souidi, M. Riottot, J.C. Bouthegourd, C. Serougne & J.C. Martin (2004). Lipid atherogenic risk markers can be more favourably influenced by the *cis*-9, *trans*-11-octadecadienoate isomer than a conjugated linoleic acid mixture or fish oil in hamsters. British Journal of Nutrition, 91, 191-199.

Vesper, H., E.M. Schmelz, M.N. Nikolova-Karakashian, D.L. Dillehay, D.V. Lynch & A.H. Merrill (1999). Sphingolipids in food and the emerging importance of sphingolipids to nutrition. Journal of Nutrition, 129, 1239-1250.

Voorrips, L.E., H.A.M. Brants, A.F.M. Kardinal, G.J. Hiddink, P.A. van den Brandt & R.A. Goldbohn (2002). Intake of conjugated linoleic acid, fat, and other fatty acids in relation to postmenopausal breast cancer: The Netherlands cohort study on diet and cancer. American Journal of Clinical Nutrition, 76, 873-882.

Warensjo, E., J.-H. Jansson, L. Berglund, K. Boman, B. Ahren, L. Weinehall, B. Lindahl, G. Hallmans & B. Vessby (2004). Estimated intake of milk fat is negatively associated with cardiovascular risk factors and does not increase the risk of a first acute myocardial infarction. A prospective case-control study. British Journal of Nutrition, 91, 635-642.

Watkins, B.A. & Y. Li (2003). CLA in functional food: Enrichment of animal products. In: J.-L. Sébébio, W.W. Christie & R.O. Adlof (eds.) Advances in Conjugated Linoleic Acid Research. Volume 2, pp. 174-188, AOCS Press, Champaign, IL.

White, S.L., J.A. Bertrand, M.R. Wade, S.P. Washburn, J.T. Green & T.C. Jenkins (2001). Comparison of fatty acid content of milk from Jersey and Holstein cows consuming pasture or a total mixed ration. Journal of Dairy Science, 84, 2295-2301.

Whitlock, L.A., D.J. Schingoethe, A.R. Hippen, K.F. Kalscheur, R.J. Baer, N. Ramaswamy & K.M. Kasperson (2002). Fish oil and extruded soybeans fed in combination increase conjugated linoleic acids in milk of dairy cows more than when fed separately. Journal of Dairy Science, 85, 234-243.

Willett, W.C., M.J. Stampfer, J.E. Manson, G.A. Colditz, F.E. Speizer, B.A. Rosner, L.A. Sampson & C.H. Hennekens (1993). Intake of *trans* fatty acids and risk of coronary heart disease among women. Lancet, 341, 581-585.

Williams, C.M. (2000). Dietary fatty acids and human health. Annual Zootechnology, 49, 165-180.

Wilson, T.A., R.J. Nicolosi, M. Chrysam & D. Kritchevsky (2000). Conjugated linoleic acid reduces early aortic atherosclerosis greater than linoleic acid in hypercholesterolemic hamsters. Nutritional Research, 20, 1795-1805.

Wolff, R.L. & D. Precht (2002). A critique of 50-m CpSil 88 capillary columns used alone to assess *trans*-unsaturated FA in foods: The case of the TRANSFAIR study. Lipids, 37, 627-629.

Yeung, C.H.T., L. Yang, Y. Huang, J. Wang & Z.Y. Chen (2000). Dietary conjugated linoleic acid mixture affects the activity of intestinal acyl coenzyme A: cholesterol acyltransferase in hamsters. British Journal of Nutrition, 84, 935-941.

Yu, Y., P.H. Correll & J.P. Vanden Heuvel (2002). Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: Evidence for a PPAR gamma-dependent mechanism. Biochimica Biophysica Acta, 1581, 89-99.

Yurawecz, M.P., N. Sehat, M.M. Mossoba, J.A.G. Roach, J.K.G. Kramer & Y. Ku (1999). Variations in isomer distribution in commercially available conjugated linoleic acid. Fett/Lipid, 101, 277-282.

Does cow's milk enhance linear growth: evidence from developing and industrialized countries

C. Hoppe[1], C. Mølgaard[1,2] and K. F. Michaelsen[1,2]
[1]Department of Human Nutrition, The Royal Veterinary and Agricultural University and Centre for Advanced Food Studies, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark
[2]Pediatric Nutrition Unit, Copenhagen University Hospital, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen Ø, Denmark

Abstract

Stunting is widespread in many developing countries, and is associated with poorer mental development, increased risk of infectious diseases and higher prevalence of cardiovascular disease. Observational and intervention studies from developing countries show that animal foods and especially cow's milk have a stimulating effect on linear growth, suggesting that animal foods provide micronutrients and high quality protein. Whether a positive association between intake of animal protein and linear growth also exists in industrialised countries, where intake of protein typically is considerably above requirements has not been clear. However, some studies indicate that protein intake and protein quality may also have a regulatory effect on growth in populations with protein intake above requirements. In our studies in well-nourished children, protein intake was positively associated with height and with the growth factor IGF-I. However, when protein intake was divided into animal and vegetable protein, and further into milk and meat, height and IGF-I were positively associated with milk, and not with vegetable protein and meat, suggesting that some milk compounds and not the amount of protein in milk have a stimulating effect on IGF-I and thereby growth. The milk constituents responsible for the apparent growth promoting effect have not yet been identified and the underlying mechanisms are not completely elucidated.

Keywords: IGF-I, cow's milk, stunting, linear growth

Introduction

Many studies from developing countries have shown that animal foods and especially cow's milk when given to infants and children have a stimulating effect on linear growth. More recent studies have suggested that cow's milk can also stimulate linear growth in a well-nourished population. The aim of this paper is to give a short overview of studies of the association between cow's milk intake and linear growth, to describe our own studies of the association between cow's milk intake and insulin-like growth factor I (IGF-I) levels and linear growth in Danish children, and to discuss some of the possible mechanisms behind a growth stimulating effect of cow's milk.

Populations with marginal or poor nutritional status

Stunting is defined as below two standard deviations of the median height of a reference population. The phenomenon is widespread in many developing countries and global estimates

show that more than 25% of children below five years are stunted (De Onis *et al.,* 2004). Stunting is associated with a poorer mental development, with increased risk of infectious diseases, with a higher prevalence of cardiovascular disease and a reduced work performance as an adult. Prevention and treatment of stunting, therefore, is a high priority.

In populations with a marginal or poor nutritional status increased intake of animal foods has shown to stimulate both weight gain and linear growth. A plausible cause is that animal foods provide micronutrients and high quality protein. However, in most populations the amount of protein intake is sufficient according to recommendations. Even if the protein intake is mainly from vegetable foods the quality of the protein intake is often reasonable, if it comes from a mixture of cereals, pulses and vegetables. Therefore, it is more likely that it is other characteristics of animal foods that are important for growth. Many studies from developing countries show that milk has a pronounced effect on linear growth. These include several intervention studies from developing countries quoted in a review of the effect of nutrition interventions (ACC/SCN, 2001). Some of these studies suggest that the effect of milk on linear growth is more pronounced than the effect of other animal foods. In an analysis of Demographic and Health Survey data of children from 12 to 36 months from seven countries in Central and South America, Ruel analysed the association between height and intake of milk, meat, and egg/fish/poultry (Ruel, 2003). Milk intake was significantly associated with higher height-for-age Z-scores in all seven data sets, whereas meat and egg/fish/poultry were only associated with height in one of the data sets. In the famous Boyd-Orr study from Scotland from the beginning of the last century the effect of milk given to school children was examined (Orr, 1928; Leighton and Clark, 1929). Children aged five to 14 years were randomized for one year to interventions either with whole milk, skimmed milk, biscuits with energy content like skimmed milk or no supplement. The two milk groups showed a higher increase in height than the other two groups. It was unclear to what degree these children were malnourished at the onset of the study.

Well-nourished populations

Whether a positive association between intake of animal protein and linear growth also exists in industrialised countries, where intake of protein typically is high, has not been clear. A protein intake below the physiological needs will result in reduced growth, but some studies indicate that protein intake and protein quality may also have a regulatory effect on growth in populations with protein intake above the requirement. Generally, the protein intake in children in industrialised countries is considerably above the requirement. During the period of complementary feeding, when infants shift from a diet based on breast milk or formula to a diet based on family foods, there is a dramatic increase in protein intake from a protein intake at about 1 g/kg body weight to an intake of 3-4 g/kg body weight, which for the average infant is 3-4 times as high as their physiological requirement. If an infant eats a typical family food with a protein energy percentage (PE%) of 15, the daily protein intake will be about 3.4 g/kg body weight. There is, however, a wide variation, and some infants have an intake of 5-6 g/kg (Rolland-Cachera *et al.,* 1999).

Several studies indicate that children who do not drink milk tend to be shorter than those who do. Black *et al.,* observed that 50 children aged 3-10 years from New Zealand, who avoid drinking cow's milk, were shorter than children drinking milk (Black *et al.,* 2002). Also children with

lactose intolerance (Stallings *et al.,* 1994) and with an allergy to milk (Paganus *et al.,* 1992; Isolauri *et al.,* 1998) are shorter than the population average.

In a randomised intervention trial with milk, Cadogan *et al.* found that supplementation with one pint of milk for 18 months tended to increase concentrations of IGF-I in 83 healthy, well-nourished 12-year-old girls who had a low habitual milk intake (~150 ml/d), in comparison with a control group (Cadogan *et al.,* 1997). The effect was significant after adjustment for pubertal status. However, the increase in IGF-I was not accompanied by a significant increase in height. The height increase was 6% in the intervention group, and 5% in the control group.

Own studies

In the Copenhagen Cohort Study (Michaelsen, 1997), 142 healthy Danish term singleton infants were followed closely from birth to one year of age, and at 10 years of age 105 children completed a follow up. The mean (SD) protein intake at nine months was 2.7 (0.7) g/kg/d and 2.7 (0.9) g/kg/d for girls and boys, respectively. At 10 years of age, the mean (SD) protein intake was 2.2 (0.5) g/kg/d and 2.4 (0.5) g/kg/d. We found with partial correlations, controlled for sex, that protein intake was positively associated with both length ($r=0.37$, $P<0.001$) and IGF-I ($r=0.30$, $P<0.05$) at nine months of age (Hoppe *et al.,* 2004a). At 10 years of age, current protein intake was positively associated with height ($r=0.35$, $P<0.001$), but not with IGF-I. Also, positive associations (tracking) of length/height ($r=0.53$, $P<0.001$), IGF-I ($r=0.27$, $P<0.03$) and protein energy percentage ($r=0.28$, $P<0.01$) were observed between infancy and late childhood. Furthermore, protein intake in infancy was positively associated with height in late childhood ($r=0.28$, $P<0.01$), suggesting that early protein intake – also above physiological requirements – may stimulate linear growth. In as much as a positive association between protein intake and circulating IGF-I in infancy, and tracking of IGF-I from infancy to late childhood was observed, the probable growth stimulating effect of a high protein intake might partly be through circulating IGF-I. IGF-I receptors are found in proliferating bone chondrocytes, and IGF-I itself stimulates synthesis of collagen and proteoglycans (Loveridge and Noble, 1994).

In an observational study of 90 healthy 2.5 year old Danish children we examined the associations between protein intake, s-IGF-I concentrations, and height (Hoppe *et al.,* 2004b). The protein intake was close to what has been found in other studies of the same age group. The 10^{th}, 50^{th}, and 90^{th} percentiles of protein intakes were 2.4, 2.9 and 4.0g per kg body weight per day. Sixty three percent of the protein intake came from animal foods. The mean (SD) intake of milk was 410 (179) ml/day in girls and 360 (130) ml/day in boys and the mean intake of meat was 34 (16) g/day in girls and 39 (26) g/day in boys. In multiple linear regressions with adjustment for sex and weight, height was positively associated with intakes of animal protein and milk, but not with those of vegetable protein or meat (Table 1). The association between milk intake and height is shown in Figure 1. Not surprisingly there was also a significant positive association between height and s-IGF-I ($p=0.02$).

These data show that milk intake was positively associated with s-IGF-I concentrations and height in this group of well-nourished Danish children with a protein intake far above the physiological requirements. The effect was quite strong as an increase in milk intake from 200 to 600 ml/d

Table 1. Effect estimates (B±S.E.) for multiple linear regressions between dietary variables (animal protein, vegetable protein, milk, and meat) with serum insulin-like growth factor I (s-IGF-I) and height. All analyses were performed with adjustment for sex and body weight. From a cross sectional study of 90 healthy Danish 2.5 year old children (Source: Hoppe et al.*, 2004b)*

	IGF-I (ng/mL)	**Height (cm)**
Animal protein (g/d)	1.4 ± 0.53 (*P*=0.013)	0.10 ± 0.038 (*P*=0.010)
Vegetable protein (g/d)	0.12 ± 1.00 (*P*=0.909)	-0.021 ± 0.069 (*P*=0.759)
Milk (g/d)	0.049 ± 0.024 (*P*=0.045)	0.0047 ± 0.002 (*P*=0.007)
Meat (g/d)	0.15 ± 0.17 (*P*=0.368)	-0.012 ± 0.012 (*P*=0.332)

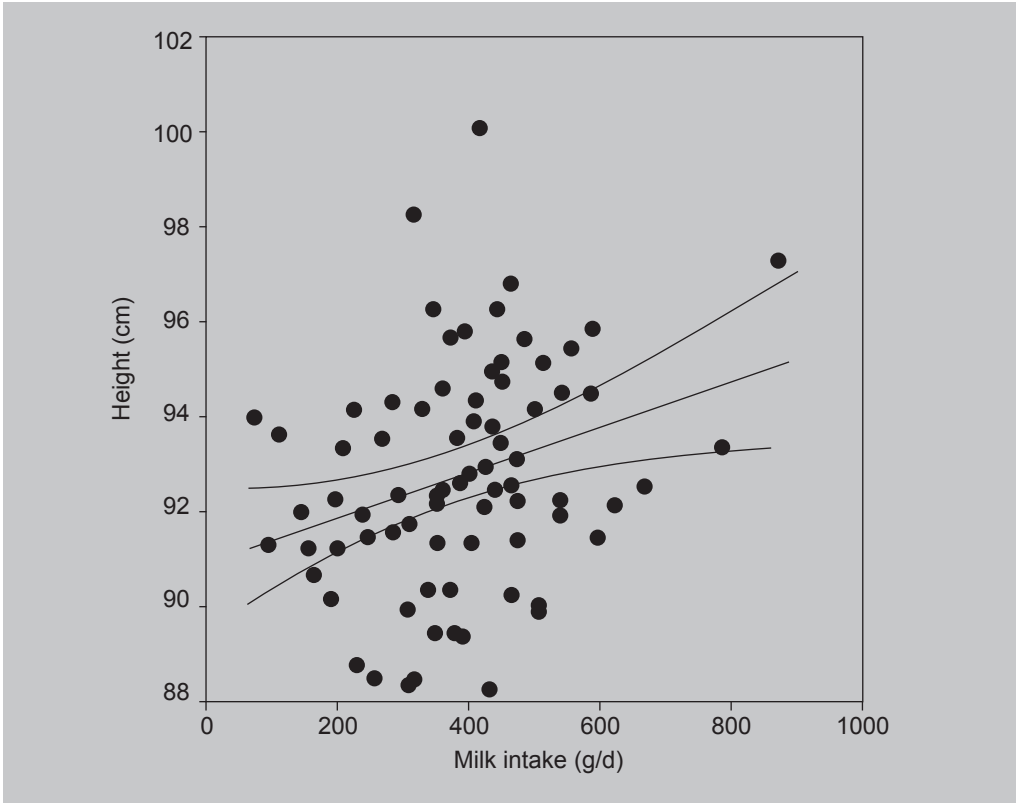

Figure 1. Height as a function of milk intake in 90 2.5-y-old children. The fitted regression line with 95% confidence intervals are shown (Source: Reproduced with permission by the American Journal of Clinical Nutrition. © Am J Clin Nutr. from Hoppe et al.*, 2004b).*

corresponded to a 30% increase in circulating s-IGF-I. Although it is an observational study, it suggests that some milk compounds have a stimulating effect on s-IGF-I and thereby growth.

We have also examined the association between milk intake and IGF-I in a short term intervention study of 24 prepubertal eight-year-old boys (Hoppe *et al.,* 2004c). They were given a one week intervention with a high intake of either milk or meat. They were asked to take either 1.5l of skimmed milk or the same amount of animal protein as 250g low fat meat. The remaining diet they could choose freely. At baseline and after seven days, the diet was registered (3-day weighed records) and s-IGF-I and s-IGFBP-3 (RIA) and s-glucose and s-insulin were determined after an overnight fast. Protein intake increased from a level of 2.3 g/kg/day by 61% in the milk group to 4.0 g/kg/day and by 54% in the meat group to 3.8 g/kg/day. The high milk intake increased concentrations of s-IGF-I by 19% ($P<0.001$) and the s-IGF-I/s-IGFBP-3 ratio by 13% ($P<0.0001$). More than 95% of circulating IGF-I is bound to IGFBP-3 in a ternary complex, which is not available to the tissues, and the molar ratio of IGF-I to IGFBP-3 may reflect free, biologically active IGF-I in circulation (Juul, 2003). There were no increases in the meat group. Thus, a high intake of milk and not meat, increased concentrations of s-IGF-I and s-IGF-I/s-IGFBP-3 significantly. The increase in protein intake in this short intervention was very high, but the level of protein intake during the intervention - expressed as gram per kg body weight per day - was close to the level seen in a large proportion of infants and young children during the complementary feeding period. During this period the average intake is typically around 3-4g/kg body weight, with some children having a considerably higher intake (Rolland-Cachera *et al.,* 1999).

We also examined the effect on s-insulin, s-glucose, and s-amino acids (Hoppe *et al.,* 2005). A high animal protein intake results in higher serum concentrations of branched chain amino acids (BCAA; leucine, isoleucine and valine), which are suggested to stimulate insulin secretion (Axelsson *et al.,* 1989). Furthermore, milk possesses some postprandial insulinotrophic effect that is not related to its carbohydrate content (Ostman *et al.,* 2001; Liljeberg Elmstahl and Bjorck 2001). In relation to linear growth insulin secretion may also be of interest. Insulin is associated with growth of the foetus and might also play a role in regulation of growth after birth. In the milk-group, fasting s-insulin concentrations doubled. In the meat-group, there was no increase in insulin. As the BCAAs increased similarly in both groups, stimulation of insulin secretion through BCAAs is not supported. Our results indicate that a short-term high milk, but not meat, intake increased insulin secretion. However, the long-term consequences of this, regarding both growth and glucose–insulin metabolism, are unknown.

Breast milk and infant formula

Infants fed milk formula, based on cow's milk, grow at a higher rate than breastfed infants, especially during the last half of infancy (Dewey, 1998). The difference is most evident for weight, but in several studies there is also a significant difference for linear growth. On the other hand, there is no difference for head circumference. The difference in growth velocity could be explained by differences in protein quality or amount, as there is a higher protein content in formula than in breast-milk. There are, however, many other differences between breast-fed and formula-fed infants than protein intake, which could explain the differences in growth, such as down-regulation of energy intake by breast-fed infants, or differences in complementary feeding

between breast-fed and weaned infants, either due to self-selection or because breast-feeding mothers provide other complementary foods.

Milk and IGF-I in adults

Several observational studies have found an association between protein intake and circulating IGF-I in adults. A positive association between protein intake and IGF-I concentrations was found in women (Holmes *et al.,* 2002) and in men, concentrations of IGF-I was higher in the highest tertile of milk intake than in the lowest tertile (Ma *et al.,* 2001). Recently, Giovannucci *et al.* (2003) found, that the major sources of animal protein, including milk, fish, and poultry, but not red meat, were associated with higher IGF-I concentrations. Conversely, Kaklamani *et al.* (1999) found that IGF-I concentrations were positively associated with intake of red meat and not with intake of milk and milk products. Furthermore, mean IGF-I concentration was 13% lower in vegan women compared with meat-eaters and vegetarians, but no difference was detected regarding IGFBP-3 (Allen *et al.,* 2002).

Possible mechanisms

It seems that milk does have a growth promoting effect, possibly through a stimulation of the growth factors IGF-I and insulin in plasma of the milk consuming individuals. The probable growth stimulating constituents of milk are unknown.

It has been hypothesised that an increase in intake of animal protein would influence, through increased concentrations of BCAAs, fasting concentrations of insulin. In our intervention study with eight-year-old boys, a high intake of milk more than doubled fasting serum insulin concentrations. In the meat-group, however, there was no increase in insulin. As the concentrations of BCAAs increased similarly in the two groups, the results from this intervention study do not support that insulin secretion was stimulated through the BCAAs (Hoppe *et al.,* 2005).

The effect of milk on IGF-I could be caused by calcium or other minerals such as zinc in milk. The effect of calcium from milk on IGF-I may differ from the effect of other calcium supplements as Wastney *et al.* (2000) found no differences in IGF-I concentrations in adolescent girls after controlled diets with high (47 mmol/d) and low (21 mmol/d) intakes of calcium as calcium citrate malate, in a cross-over design.

Even though cow's milk contains IGF-I that is structurally identical to human IGF-I (Daxenberger *et al.,* 1998; Juskevich and Guyer, 1990) in a concentration of approximately 30 ng/ml (Outwater *et al.,* 1997), and rat studies suggest intact absorption (Xian *et al.,* 1995), it is the general belief that IGF-I will not retain bioactivity when delivered orally because of rapid proteolysis in the upper gut of humans (Daxenberger *et al.,* 1998; Juskevich and Guyer 1990). Cow's milk is rich in other trophic factors such as hormones and cytokines, growth factors and many bioactive peptides (Playford *et al.,* 2000), which could also play a role.

We speculate that the effect is related to bioactive factors present in milk and we are at present studying the effects of casein and whey with and without milk minerals on IGF-I and insulin.

About 80% of cow's milk proteins are casein and 20% are whey. When the pH is decreased, casein proteins clot. Hence, the acidity in the stomach makes casein aggregate into a gel, whereas whey remains soluble. In a study of the effect of lactose-equivalent dietary sources of protein on concentrations of postprandial insulin, the insulin response to a whey meal was more pronounced than that to milk meal (Nilsson *et al.,* 2004), which indicate that the insulinotrophic components may be connected to the soluble milk proteins.

Linear growth and non-communicable diseases

The long-term implications of increased growth and increased concentration of circulating IGF-I and insulin caused by a high intake of protein or milk are not known. However, increase in linear growth is liable to have both positive and negative effects. A high growth velocity has traditionally been regarded as beneficial and associated with good health (Tanner, 1992) and a reduced growth velocity has been associated with adverse health and development outcomes (Barker, 1992). Many studies have found that higher adult stature is associated with better health (Silventoinen *et al.,* 1999), a lower overall mortality (Waaler 1984), and reduced risk of certain non-communicable diseases, such as coronary heart disease (Peck and Vagero, 1989). Conversely, other studies have found that tallness in both childhood (Gunnell *et al.,* 1998) and adulthood (Smith *et al.,* 1998) is related to higher cancer risk. And a high growth velocity during certain periods of infancy and childhood might result in an increased risk of non-communicable diseases, *e.g.* certain cancer forms, such as breast, ovary and prostate cancers (Micozzi, 1993), hypertension (Rona *et al.,* 1996) and coronary heart disease (Fall *et al.,* 1995). The relationship between growth velocity and risk of disease later in life is not simple, as Eriksson *et al.* (2001) found that low growth velocity during infancy was associated with increased risk of coronary heart disease, whereas after the age of one year, a high growth velocity was associated with further increase in risk.

IGF-I and non-communicable diseases

Numerous epidemiological studies have found associations between IGF-I concentrations and some non-communicable diseases in adults. Low concentrations of IGF-I may be associated with increased rates of cardiovascular disease (Juul, 2003), whereas high concentrations of IGF-I are associated with an increased risk of prostate (Gunnel *et al.,* 2003) and breast cancer (Outwater *et al.,* 1997). A detailed discussion of these findings is beyond the focus of this paper.

Conclusion

There is considerable evidence that cow's milk can stimulate linear growth and it is likely that it happens through a stimulation of the IGF-Is. The growth stimulating effect seems to take place also in well-nourished populations where nutritional deficiencies are not likely. The traditional concept that growth is optimal, if all nutrients are available is, therefore, too simplistic. A hormonal modulation of growth velocity is likely to be at least part of the growth promoting effects of milk. We suggest that the active components in milk could be bioactive peptides, a combination of certain amino acids or a combination of protein and minerals.

Stunting is widespread in many developing countries and has severe long-term consequences. Adding cow's milk or milk powder to the diet of stunted children might be an effective and relatively inexpensive way to improve linear growth, and thereby, reduce morbidity and improve development. While the infant is still breast fed it is important not to give cow's milk as a drink not to interfere with breast milk volume. However, milk can be added to porridges and other foods. In industrialised countries the long-term consequences of an increased linear growth velocity during childhood in well-nourished children are likely to be both positive and negative. Understanding the mechanisms through which milk influences linear growth is important and could improve our understanding on how to prevent and treat stunting. If we understand which milk components are responsible for the growth stimulation, we might be able to optimize feeding in situations where we use milk components like infant formula, tube feeding and therapeutic feeding of malnourished children.

References

ACC/SCN (2001). What works? A review of the efficacy and effectiveness of Nutrition interventions. Allen LH and Gillespie SR: Geneva, in collaboration with the Asean Development Bank, Manila.

Allen, N.E., P.N. Appleby, G.K. Davey, R. Kaaks, S. Rinaldi & T.J. Key (2002). The associations of diet with serum insulin-like growth factor I and its main binding proteins in 292 women meat-eaters, vegetarians, and vegans. Cancer Epidemiol Biomarkers Prev, 11,1441-1448.

Axelsson, I.E., S.A. Ivarsson & N.C. Raiha (1989). Protein intake in early infancy: effects on plasma amino acid concentrations, insulin metabolism, and growth. Pediatr Res., 26(6), 614-617.

Barker, D.J.P. (1992). Fetal and infant origin of adult disease. London: British Medical Association,.

Black, R.E., S.M. Williams, I.E. Jones & A. Goulding (2002). Children who avoid drinking cow milk have low dietary calcium intakes and poor bone health. Am J Clin Nutr, 76, 675-680.

Orr, J.B. (1928). Milk consumption and the growth of school-children. Lancet, i, 202-203.

Cadogan, J., R. Eastell, N. Jones & M.E. Barker (1997). Milk intake and bone mineral acquisition in adolescent girls: randomised, controlled intervention trial. BMJ, 315, 1255-1260.

Daxenberger, A., B.H. Breier & H. Sauerwein (1990). Increased milk levels of insulin-like growth factor 1 (IGF-1) for the identification of bovine somatotropin (bST) treated cows. Analyst 1998, 123, 2429-2435.

Juskevich, J.C. & C.G. Guyer (1990). Bovine growth hormone: human food safety evaluation. Science, 249, 875-884.

Dewey, K.G. (1998). Growth characteristics of breast-fed compared to formula-fed infants. Biol Neonate, 74, 94-105.

Eriksson, J.G., T. Forsen, J. Tuomilehto, C. Osmond & D.J. Barker (2001). Early growth and coronary heart disease in later life: longitudinal study. BMJ, 322, 949-953.

Fall, C.H., M. Vijayakumar, D.J. Barker, C. Osmond & S. Duggleby (1995). Weight in infancy and prevalence of coronary heart disease in adult life. BMJ, 310, 17-19.

Giovannucci, E., M. Pollak, Y. Liu, E.A. Platz, N. Majeed, E.B. Rimm & W.C. Willett (2003).Nutritional Predictors of Insulin-like Growth Factor I and Their Relationships to Cancer in Men. Cancer Epidemiol Biomarkers Prev, 12, 84-89.

Gunnell, D.J., G.D. Smith, J.M. Holly & S. Frankel (1998). Leg length and risk of cancer in the Boyd Orr cohort. BMJ, 317, 1350-1351.

Gunnell, D., S.E. Oliver, T.J. Peters, J.L. Donovan, R. Persad, M. Maynard, D. Gillatt, A. Pearce, F.C. Hamdy, D.E. Neal & J.M. Holly (2003). Are diet-prostate cancer associations mediated by the IGF axis? A cross-sectional analysis of diet, IGF-I and IGFBP-3 in healthy middle-aged men. Br J Cancer, 88(11), 1682-1686

Holmes, M.D., M.N. Pollak, W.C. Willett & S.E. Hankinson (2002). Dietary correlates of plasma insulin-like growth factor I and insulin-like growth factor binding protein 3 concentrations. Cancer Epidemiol Biomarkers Prev, 11, 852-861.

Hoppe, C., C.Mølgaard, B.L. Thomsen, A. Juul & K.F. Michaelsen (2004a). Protein intake at 9 mo is associated with body size but not with body fat in 10-y-old Danish children. Am J Clin Nutr, 79, 494-501.

Hoppe, C., T.R. Udam, L. Lauritzen, C. Mølgaard, A. Juul & K.F. Michaelsen (2004b). Animal protein intake, serum insulin-like growth factor I, and growth in healthy 2.5-y-old Danish children. Am J Clin Nutr, 80, 447-452.

Hoppe, C., C. Mølgaard, A. Juul & K.F. Michaelsen (2004c). High intakes of skimmed milk, but not meat, increase serum IGF-I and IGFBP-3 in eight-year-old boys. Eur J Clin Nutr, 58, 1211-1216.

Hoppe, C., C. Mølgaard, A. Vaag, V. Barkholt & K.F. Michaelsen (2005). High intakes of milk, but not meat, increase s-insulin and insulin resistance in 8-year-old boys. Eur J Clin Nutr, 59, 393-398.

Isolauri, E., Y. Sutas, M.K. Salo, R. Isosomppi & M. Kaila (1998). Elimination diet in cow's milk allergy: risk for impaired growth in young children. J Pediatr, 132, 1004-1009.

Juul, A. (2003). Serum levels of insulin-like growth factor I and its binding proteins in health and disease. Growth Horm IGF Res, 13, 113-170

Kaklamani, V.G., A. Linos, E. Kaklamani, I. Markaki, Y. Koumantaki & C.S. Mantzoros (1999). Dietary fat and carbohydrates are independently associated with circulating insulin-like growth factor 1 and insulin-like growth factor-binding protein 3 concentrations in healthy adults. J Clin Oncol, 17, 3291-3298.

Leighton, G. & M.L. Clark (1929). Milk comsumption and the growth of schoolchildren. Lancet, 40-43.

Liljeberg Elmstahl, H. & I. Bjorck (2001). Milk as a supplement to mixed meals may elevate postprandial insulinaemia. Eur J Clin Nutr. Nov, 55(11), 994-999.

Loveridge, N. & B.S. Noble (1994). Control of longitudinal growth: the role of nutrition. Eur J Clin Nutr, 48, 75-84.

Ma, J., E. Giovannucci, M. Pollak, J.M. Chan, J.M. Gaziano, W. Willett & M.J. Stampfer (2001). Milk intake, circulating levels of insulin-like growth factor-I, and risk of colorectal cancer in men. J Natl Cancer Inst, 93, 1330-1336.

Micozzi, M.S. (1993). Functional consequences from varying patterns of growth and maturation during adolescence. Horm Res, 39 Suppl 3, 49-58.

Michaelsen, K.F. (1997). Nutrition and growth during infancy. The Copenhagen Cohort Study. *Acta Paediatr Suppl* 420, 1-36.

Nilsson, M., M. Stenberg, A.H. Frid, J.J. Holst & I.M. Bjorck (2004). Glycemia and insulinemia in healthy subjects after lactose-equivalent meals of milk and other food proteins: the role of plasma amino acids and incretins. Am J Clin Nutr. Nov, 80(5), 1246-1253.

de Onis, M., M. Blossner, E. Borghi, R. Morris & E.A. Frongillo (2004). Methodology for estimating regional and global trends of child malnutrition. Int J Epidemiol, 33, 1260-1270.

Ostman, E.M., H.G. Liljeberg Elmstahl & Bjorck I.M. (2001). Inconsistency between glycemic and insulinemic responses to regular and fermented milk products. Am J Clin Nutr, 74, 96-100.

Outwater, J.L., A. Nicholson & N. Barnard (1997). Dairy products and breast cancer: the IGF-I, estrogen, and bGH hypothesis. Med Hypotheses, 48, 453-461.

Paganus, A., K. Juntunen-Backman & E. Savilahti (1992). Follow-up of nutritional status and dietary survey in children with cow's milk allergy. Acta Paediatr, 81, 518-521.

Peck, A.M. & D.H. Vagero (1989). Adult body height, self perceived health and mortality in the Swedish population. J Epidemiol Community Health, 43, 380-384.

Playford R.J., C.E. Macdonald & W.S. Johnson (2000). Colostrum and milk-derived peptide growth factors for the treatment of gastrointestinal disorders. Am J Clin Nutr, 72, 5-14.

Rolland-Cachera, M.F., M. Deheeger & F. Bellisle (1999). Increasing prevalence of obesity among 18-year-old males in Sweden: evidence for early determinants. Acta Paediatr, 88, 365-367.

Rona, R.J., S. Qureshi & S. Chinn (1996). Factors related to total cholesterol and blood pressure in British 9 year olds. J Epidemiol Community Health, 50, 512-518.

Ruel, M.T. (2003). Milk intake is associated with better growth in Latin America: Evidence from the Demographic and Health Surveys. The FASEB Journal, 17, A119 (abstract 768.2)

Silventoinen, K., E. Lahelma & O. Rahkonen (1999). Social background, adult body-height and health. Int J Epidemiol, 28, 911-918.

Smith G.D., M. Shipley & D.A. Leon (1998). Height and mortality from cancer among men: prospective observational study. BMJ, 317, 1351-1352.

Stallings, V.A., N.W. Oddleifson, B.Y. Negrini, B.S. Zemel & R. Wellens (1994). Bone mineral content and dietary calcium intake in children prescribed a low-lactose diet. J Pediatr Gastroenterol Nutr, 18, 440-445.

Tanner, J.M. (1992). Growth as a measure of the nutritional and hygienic status of a population. Horm Res, 38 Suppl 1, 106-115.

Waaler, H.T. (1984). Height, weight and mortality. The Norwegian experience. Acta Med Scand Suppl, 679, 1-56.

Wastney, M.E., B.R. Martin, M. Peacock, D. Smith, X.Y. Jiang, L.A. Jackman & C.M. Weaver (2000). Changes in calcium kinetics in adolescent girls induced by high calcium intake. J Clin Endocrinol Metab, 85, 4470-4475.

Xian, C.J., C.A. Shoubridge & L.C. Read (1995). Degradation of IGF-I in the adult rat gastrointestinal tract is limited by a specific antiserum or the dietary protein casein. J Endocrinol, 146, 215-225.

Part IX: Workshop reports

The use of ruminants in less developed countries and the priorities within ruminant physiology research to assist in development

Chaired by J. Madsen and T. Hvelplund

Discussion paper — Ruminants in agricultural development: where is the future for animal physiologists?

J. Madsen
Department of Large Animal Sciences, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark

Summary

A reference was made to the ISRP 1994 in Willingen, Germany, where E. R. Ørskov initiated a workshop in relation to problems in developing countries, based on the fact that only few of the presentations on the ISRP symposiums relate to specific problems in developing countries. The situation has been improved and quite a lot of posters at the ISRP2004 in Copenhagen relates to ruminant production in the tropics and developing countries.

The use of the ruminants in developing countries is more diverse than in industrialised countries. Ruminants are kept for food production and income generation, but in addition they are also used for draught purposes in ploughing, weeding, transport etc. and in general they compensate or substitute many of the services available in more developed societies. The livestock bank most directly substituting unreliable or unavailable banks for saving and loans. The livestock also substitute insurance companies and social security by providing available capital when unexpected expenses arise. They serve as food security as they survive and can be sold or eaten in periods where the harvest of grain and other crops fail. They serve as relative readily available capital and in addition livestock display wealth, especially the larger livestock. The manure from the ruminants is also highly appreciated in developing countries whereas the manure has become in surplus is more of the industrialised countries. This diversity in use of ruminants has to be taken more serious in the future within planning of development assistance and policy making, if substantial improvements in the use of ruminants for the benefit of people shall be achieved.

The feeds are different and generally the roughages are less digestible and contain less protein in the tropics compared to the temperate regions.

The breeds are different, the natural environment and housing is different, the infrastructure, organisation, education, access to finances and services, the ownership to land etc. is different.

So many things are different, - and for sure a lot of things are the same -, but the question is: Does these differences mean that we as physiologists shall have specific and different priorities in research if we want to give specific priority to ruminant production in developing countries. If so what are the specific priorities?

It was concluded that there were specific areas that should be given priority. The priority areas could be divided into technical issues and approach issues and examples were given. The workshop participants were invited to reflect on the following:
- Where are the opportunities?

- What is your experience?
- Let us hear about your hobbyhorses!

Discussion summary

The following bullet points lists the priorities as they were brought up in the discussion at the workshop. The bullet points are divided into technical issues and approach issues. The latter reflects the general feeling that the approach used in the research is of great importance. It is felt that the relevance of the applied and strategic research is best secured if there is a relation to practical farming and this relation also facilitate the use of the results from the research. It is also recognized that basic research may have to be conducted with no connection to farmers. The bullet points are not listed in order of priority.

Technical issues**:**

- Methods to improve feed value of tropical feeds.
  - Magic feed (ex. open the lignifications of feeds).
  - Screening of potential fodder plants (not only Leucenea!).
  - Mycotoxins, eg. Aflatoxine.
  - Anti nutritional factors.
- Methods to conserve feeds for the dry period.
- Easy methods to predict/measure the feed value of available feeds – and the range. (all importent nutrients).
- Rumen microorganisms with special abilities (ex. Mimosine).
- Establish biologic relations to be able to chose the right animal / ruminant for the right purpose, feed and environment.
- N and P recycling.
- Investigate how indigenous animals cope with scarcity of nutrients, water, heat stress, etc..

Approach issues:

- Holistic incl. socio-economics, policy, market etc. (interdisciplinary).
- Make literature available.
- Do not copy research.
- Collaboration with farmers incl. training of farmers (action research).
- Collaboration with NARS, NGOs and local institutions.
- Collaboration with development agencies.
- Combine research, education and training.
- Modelling should also be done on less intensive/tropical systems.
- Specific high-tech issues can be researched in an industrial country according to needs, - (and at the same time be relevant for development).

Part IX: Workshop reports

Methods used for studying particle size and digesta flow

Chaired by D.P. Poppi and A.de Vega

Discussion paper 1 — Use of image analysis for measuring particle size in feed, digesta and faeces

P. Nørgaard
Department of Animal and Veterinary Basic Sciences, The Royal Veterinary and Agricultural University, Grønnegårdsvej 3, DK-1870 Frederiksberg

Introduction

Mastication during eating and ruminating, and digestive processes reduce feed particles to smaller particles with a mean length of 1-2 mm (Poppi *et al.*, 1981). The particle size in the feed affects the passage rate and digestion of the individual particles. Likewise, the size of feed particles has an overall effect on time spent mastication, on saliva secretion, on rumen motility, characteristics of rumen contents, on fermentative processes, microbial yield and a risk for digestive disorders. The size of feed particles varies been plant species, stage of growth at harvest, anatomical fraction and processing like chopping or grinding (Mertens, 1997). The range of particle length in forages, rolled grain, finely processed concentrates, forage boli, rumen contents and faces is illustrated in Figure 1.

The size of feed particles, digesta and faeces has previously been measured by use of dry or wet sieving technique (Allen *et al.*, 1984) and more recently by use of image analysis (Luginbuhl *et al.*, 1991; Nørgaard and Bendixen, 2002; Nørgaard and Sehic, 2003; Nørgaard *et al.*, 2004). The Penn State Particle Separator has been widespread used for measurement of particle size in forage and total mixed rations (Kononoff *et al.* 2003). The particle size have been characterized by the

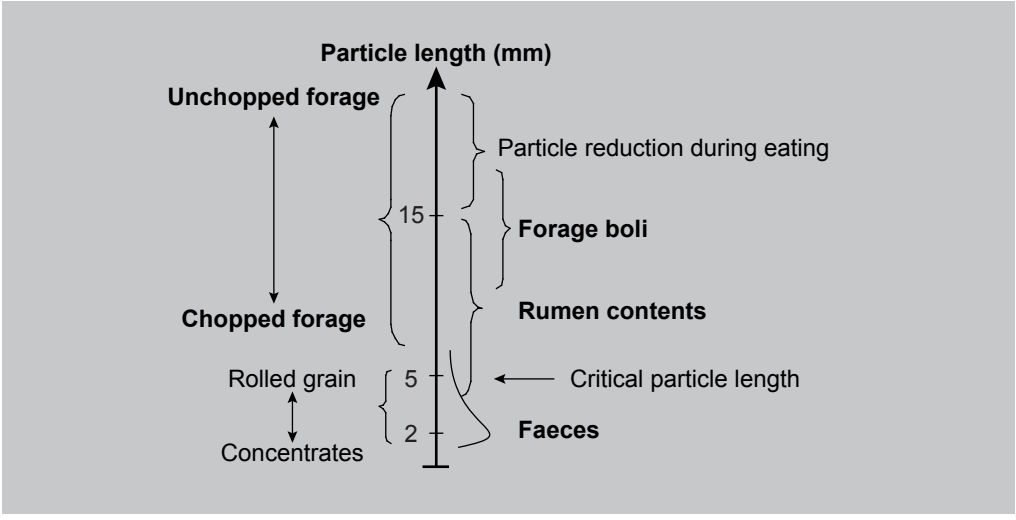

Figure 1. Range of particle lengths found in forages, concentrates, forage boli, rumen contents and faeces from cows (Sources: Nørgaard and Bendixen, 2002; Nørgaard and Sehic, 2003).

proportion of particles retained in the individual sieve fractions, from which the means size, the mean length or width can be calculated (Luginbuhl *et al.*, 1991). The distribution of particle size in processed feeds, digesta and faeces is characterized by many small particles and few large particles, which lead to a left skewed non normal distribution. The distribution of particle size has been characterized by the most frequent size value (mode), geometric mean, arithmetic median (Allen *et al.*, 1984) and the 95 percentile of the larges sizes (95_PS) values. The typical ranking of theses values in processed feed, digesta and faeces is: mode < geometric mean value < median value < arithmetic mean < 95_PS (ASAE, 1991; de Boever *et al.*, 1993; Nørgaard *et al.*, 2004).

Some of the major questions to be asked are:
What do we mean by *particle size*? Particle weight, volume, length, width or perimeter?
Why and how do we measure *particle size*? Sieving technique ? Image analysis ?
How do we express *particle size*? Mode, median, arithmetic mean? Distribution functions?

Measuring particle dimensions by use of image analysis

The particles in feed boli, rumen contents or faeces are washed in sieves or nylon bags with pore sizes of 0.01 or 0.04 mm before freeze drying as described by (Nørgaard and Sehic, 2003; Nørgaard *et al.*, 2004). The dried particles or chopped forage particles are sorted into 3 to 4 sieving fractions including the bottom bowl (B). Sub samples of 0.002 to 2 g from each sieving fraction are distributed on the scanner cowering about 1 to 5 % of the scanned area. Overlapping particles are manually rearranged before scanning at 300 to 2400 dpi depending on particle size. The individual particles are identified and their area (PA_i), length (PL_i) and width (PB_i) values are estimated from image analysis using software like ex. Image ProPlus, CyberCity.

Methods for prediction of distribution and dimension values

The arithmetic mean particle length (APL_j) in the j^{th} sieving fraction is estimated from equation (1):

$$APL_j = (\Sigma PL_{ji} \times PA_{ji}) / \Sigma PA_{ji} \quad (1)$$

The overall arithmetic mean particle length (APL) is estimated by weighting the APL_j values with the mass proportions (m_j) of the individual sieve fractions as shown in equation (2).

$$APL = \Sigma APL_j \times m_j \quad (2)$$

The geometric mean particle length in the sample is estimated from equation (3)

$$GPL = \exp(\ (\Sigma(\Sigma \ln(PL_{ji}) \times PA_{ji}) / \Sigma PA_{ji}) \times m_j) \text{ as described by Nørgaard and Sehic (2003)} \quad (3)$$

The distribution of particle length within sieving fractions is estimated using a gamma distribution $\gamma_j(PL,\alpha_n,\beta_n)$ including weighting the individual PL values with their PA value within sieving fractions. The most frequent length value (mode) within the j^{th} sieving fraction (Mo_PL_j) can be estimated from equation (4).

$$Mo\_PL_j = (\alpha_j - 1) \times \beta_j \quad (4)$$

The overall distribution of particle length $C(\gamma_j, m_j)$ is estimated as $\Sigma\gamma_j \times m_j$. The overall most frequent particle length (mode value) (Mo_PL) can be estimated by stepwise identification of the maximal $C(\gamma_j, m_j)$ value using the pdf distribution function in SAS (SAS, 2000). The overall accumulative distribution function $A(A_j, m_j)$ can be estimated as $\Sigma A_j \times m_j$, where equation (5):

$$A_j(PL,\alpha_j,\beta_j) = {}_0\int^{PL} \gamma_j(PL,\alpha_j,\beta_j) \times dPL \quad (5)$$

denotes the accumulated density function for the j^{th} sieve fraction. The overall median (MPL) and 95 percentile (95PL) values can be estimated by stepwise identification of the 0.5 and 0.95 value from $A(A_j, m_j)$ by using the cdf function in SAS (SAS, 2000).

Particle length distributions and size characteristics

Figure 2 shows a gamma distribution of particle length in grass silage chopped at a theoretical chopping length of 19 mm with an indication of the different particle size units. The most frequent length value (Mo_PL) of 18.8 mm is close to the theoretical chopping length (TCL) value of 19.1 mm, which is lower than the GPL, MPL and the APL value of 27, 29 and 34 mm, respectively. The results from the sieving analysis resulted in an APS value of 3.1 and a GPS value of 2.6 mm. These values are only 10% of the GPL and APL values, respectively. The lower APS and GPS can be explained by the fact that sieving technique often perform a sorting of particles according to their width. The arithmetic mean width (APB) and geometric mean width (GPB) were measured to 1.46 and 1.37 mm, respectively (Nørgaard and Bendixen, 2002).

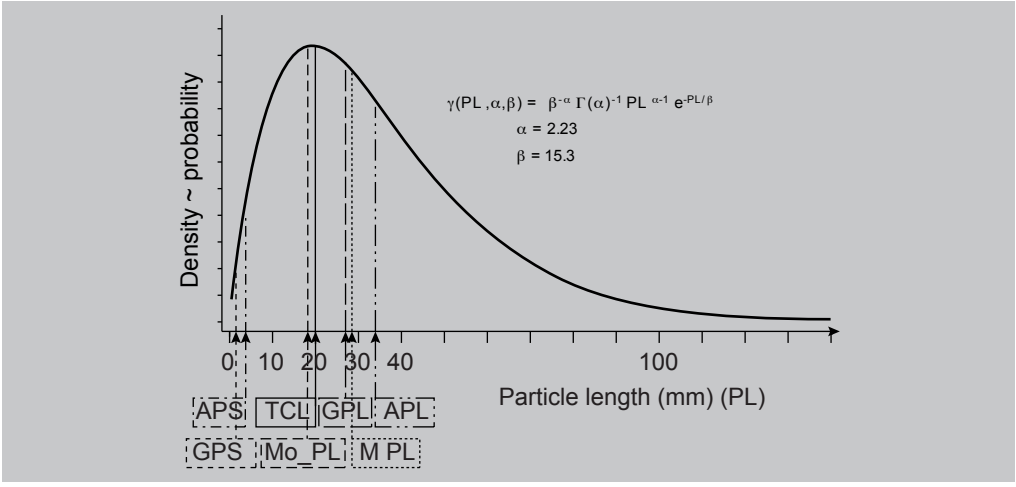

Figure 2. Gamma density plot of particle length in grass silage chopped at 19 mm theoretical chopping length (TCL) including indications of APL (arithmetic mean), GPL (geometric mean), MPL (median), Mo_PL (mode), APS (arithmetic mean particle size) and GPS (geometric particle size) values (Source: mod. Nørgaard and Bendixen, 2002).

The cumulative distribution of particle length (Figure 3) illustrates the decreasing median particle length (MPL) of 28 m in grass silage chopped at 19.1 mm TCL, 17 mm in silage boli collected at cardia, 2 mm in rumen contents and to 1 mm in faeces from Jersey cows fed grass silage. 95% of the particles in faeces, rumen contents, boli and chopped silage were longer than 6, 20, 46 and 77 mm, respectively, in cows fed grass silage chopped at 19.1 mm TCL. The critical particle length in faeces (CPL) appears to have a value of about 6 mm, which is 5 times longer than the critical particle size (CPS) value of 1.14 mm proposed by (Poppi *et al.*, 1981; Mertens, 1997; Kennedy and Poppi, 1984), but again the faeces particle are typical 5 times longer compared with their width.

Conclusions and applications

- Particle size distributions in feed, feed boli, rumen contents and faeces can be measured by use of image analysis
- Particle size distribution in digesta can be affected by the physical form of forage and grains
- An arithmetic or geometric mean particle size appears to be poor descriptors of particle size distribution in feed and digesta
- Processed feed and digesta contains many small particles and relative few large particles
- The mode, median and 95 percentile values appear as essential descriptors of the distribution of particle size in processed feed, digesta and faeces.
- Image analysis appears to be a useful tool for characterization of the physically dimensions of feed and digesta particles, and for studying particle break down along the digestive tract in different ruminant species and for comparison with other species

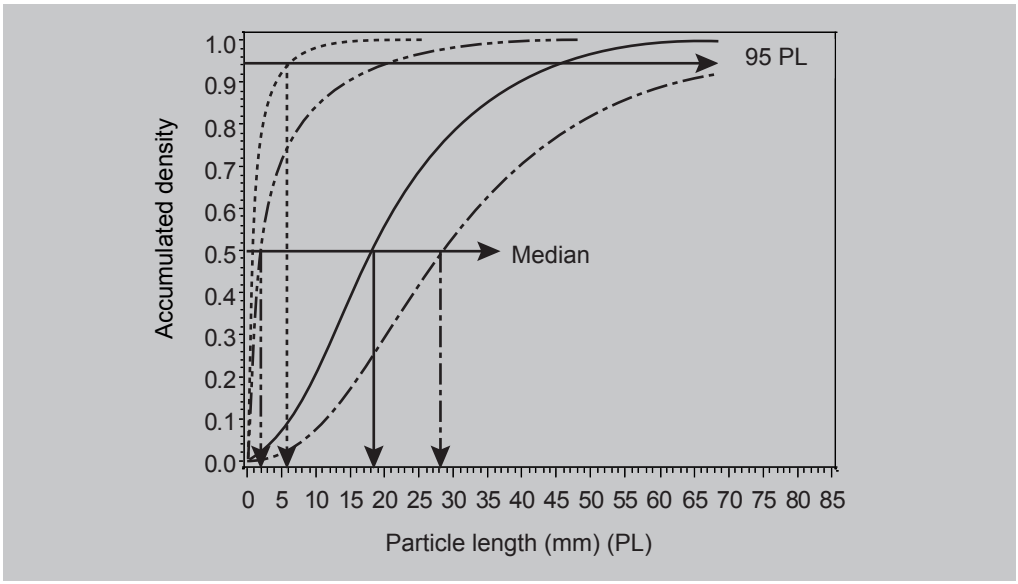

Figure 3. Cumulative distribution of particle length (PL) (mm) in faeces (- - -), rumen contents (— - - —) and boli (——) from Jersey cows fed grass silage (— - —) chopped at 19 mm theoretical chopping length including median length values (MPL) and 95 percentile (95PL) values (Mod. Nørgaard and Sehic, 2003).

References

Allen, M., J. Robbertson & P. van Soest, P. (1984). A comparison of particle size methodologies and statistical treatments. In: P.M. Kennedy (ed) Techniques in particle size analysis of feed and digesta in ruminants. Proc. of a workshop held in Banff, Alberta, Canada. Canadian society of animal science, occasional publ. no 1, 39-56.

ASAE (1991). Method of determining and expressing particle size of chopped forage materials by screening. ASAS standards, 409-411.

Boever, de J.L., A. de Smet, D.L.de Brabander & C.V. Boucque (1993). Evaluation of physical structure. 1. Grass silage. Journal of Dairy Science, 76, 140-153.

Kennedy, P. & D.P. Poppi (1984). 'Critical partical size' in sheep and cattle. In: P.M. Kennedy (ed) Techniques in particle size analysis of feed and digesta in ruminants. Proc. of a workshop held in Banff, Alberta, Canada. Canadian society of animal science, occasional publ. no 1, 170.

Kononoff, P.J., A.J. Heinrichs & D.R. Buckmaster (2003). Modification of the Penn State forage and total mixed particle separator and the effects of moisture contents on its measurements. Journal of Dairy Science, 86, 1858-1863.

Luginbuhl, J.M, D.S. Fisher, K.R. Pond & J.C. Burns (1991). Image analysis and nonlinear modeling to determine dimensions of wet-sieved, masticated forage particles. Journal of Animal Science, 69, 3807-3816.

Mertens, D., (1997). Creating a system for meeting the fibre requirements of dairy cows. Journal of Dairy Science, 80, 1463-1481.

Nørgaard, P. & B. Bendixen (2002). Particle size distribution in silage, boli, rumen content and faeces from cows fed grass silage with different theoretical chopping length. Annual Meeting of EAAP, Cairo, 6 pp

Nørgaard, P., S. Husted & H. Ranvig (2004). Effect of supplementation with whole wheat or whole oat grains on the dimensions of faeces particles from lambs. Journal of Animal and Feed Sciences, suppl. *1,* 13, 175-178.

Nørgaard, P. & A. Sehic (2003). Particle size distribution in silage, boluses, rumen content and faeces from cows fed grass silage with different theoretical chopping length. The Sixth International Symposium on the Nutrition of Herbivores 19 to 24 October, 2003, Mérida, Yucatán, México. Tropical and Subtropical Agroecosystems, 3, 457-460.

Poppi, D.P., D.J. Minson & J.H. Ternouth (1981). Studies of cattle and sheep eating leaf and stem fractions of grasses. 3. The retention time in the rumen of large feed particles. Australian Journal of Agricultural Research, 32, 123-137.

SAS, 2000. Statistical Analysis System. User Guide. Statistical Analysis Institute, Inc. Cary, NC.

Waldo, D.R., L.W. Smith, E.L.Cox, B.T. Weinland & H.L. Lucas (1971). Logarithmic normal distribution for destribution of sieved forage materials. Journal of Dairy Science, 54, 1465-1469.

Appendix

Estimations from a gamma distribution of PL:

1. $\gamma_{PL}(PL,\alpha,\beta) = \beta^{-\alpha}\,\Gamma(\alpha)^{-1}\,PL^{\alpha-1}\,e^{-PL/\beta}$, $\Gamma(\alpha) = {}_0\int^{\infty} x^{a-1}e^{-x}\,dx$
2. $\alpha = \mu^2/\sigma^2$ and $\alpha = \mu/(\mu\text{-mode})$ for $\alpha>1$
3. $\beta = \sigma^2/\mu$ and $\beta = \mu$-mode for $\alpha>1$
4. Arithmetic mean: $\mu = \alpha \times \beta$
5. Mode = $(\alpha\text{-}1)\,\beta = \mu - \sigma^2/\mu$ for $\alpha > 1$ and mode $=0$ for $\alpha \leq 1$
6. $\sigma^2 = \alpha\,\beta^2$ and $\sigma^2 = (\mu\text{-mode})\mu$ for $\alpha > 1$
7. Accumulated distribution: $A(PL,\alpha,\beta) = {}_0\int^{PL} \gamma(PL,\alpha,\beta)\,d\,PL$
8. Median : $A(Median,\alpha,\beta) = 0.5$
9. 95Percentile (95PL): $A(95PL,\alpha,\beta) = 0.95$

Estimations of particle size characteristics from sieving analysis, where the mass proportion (m_j) of particles retained in the j^{th} sieve fraction with a pore size of P_j. The particle size in sieve fraction$_j$ (PS_j) can be estimated as $\sqrt{(P_j \times P_{j+1})}$ and $PS_{\text{Botton bowel}}$ as $P_1/2$ according to (Waldo *et al.*, 1971).

10. Arithmetic mean Particle Size : $APS = \Sigma m_j \sqrt{(P_j \times P_{j+1})}$
11. Geometric mean Particle Size : $GPS = \exp(\Sigma m_j \times \ln(\sqrt{(P_j \times P_{j+1})}))$
12. Mode Particle Size $Mo\_PS = APS - (\Sigma PS_j^2 \times m_j - APS^2)/APS$
13. Median Particle Size: MPS = PS for $A(MPS,\alpha,\beta) = 0.5$
14. 95 percentile Particle Size 95PS = PS for $A(95PS,\alpha,\beta) = 0.95$

Discussion summary

Discussion followed on various applications of the image analysis. The method measures particle length and images were shown of the various particle shapes from different sample sites and feed types. Image analysis software was used to rapidly assess the images. The method is different to previous methods such as wet sieving which discriminates largely on diameter but given the concept that the rumen is a self-sieving mechanism then length would be a more interesting parameter on which to investigate escape of particles from the rumen.

The cost at present is in line with other methods of chemical analysis but sample preparation and analysis takes about 4hrs. The sample size varies from ca. 10mg for faeces and 2-4g for feed. The method currently uses freeze drying and a dry sieving procedure but there is no reason why wet samples could not be used. Currently, freeze drying fitted in with other sample processing requirements. The density of particles of different length had not been measured. Samples of maize silage, whole barley and grass silage had been investigated. The question arose as to why forages and faeces have been examined in this way. Faecal particle length and distribution is linked to factors affecting fibre digestion. Forages were being studied to get an index related to effective fibre intake by linking length distribution with chewing activities and forage intake.

There were some problems with the procedure. Very small particles could not easily be distinguished with current software. There was debate on most appropriate statistical procedures. Currently median length and 95% percentile were used and these seemed to be appropriate to assess effective length and effective fibre. It was agreed that length was a valuable addition to wet sieving as a procedure to describe particle size. There was discussion on the practical application of the method. Currently it was being used to assess effective fibre for animal health and was also used diagnostically to assess effective fibre. The method had a unique description of particle size (length) which could be applied to studies into intake regulation and rate of particle length breakdown. The critical particle size of ca. 1mm has no relationship to length as measured here and hence a new dimension of particle kinetics within the rumen could be explored.

Recommendations

A brief description of the method has been given above and some reports of its application are given in Nørgaard and Sehic (2003). The statistical procedure of calculating as described above is recommended whereupon the data may be used for animal health relating to effective fibre and also in experimental measurements of factors affecting retention time of digesta within the rumen.

Discussion paper 2 — Measurement of digesta flow entering the omasal canal

S. Ahvenjärvi
MTT Agrifood Research Finland, FIN-31600, Jokioinen, Finland

Understanding and quantification of ruminal nutrient metabolism has been largely based on digesta flow measurements. Typically, to assess forestomach digestibility digesta samples have been obtained from the duodenum. Compared with sampling from the duodenum the omasal canal provides some advantages as a sampling site. Secretion of endogenous N into the reticulorumen is lower than that into the duodenum. The omasal canal sampling technique allows the contribution of soluble N components (protein, peptides and free amino acids), microbial populations (liquid and particle associated bacteria, protozoa and fungi) and insoluble dietary N components to N flow from the reticulorumen to be determined. Sampling from the duodenum has the disadvantage that digesta leaving the abomasum has been subject to enzymatic digestion such that various N fractions cannot be distinguished.

In order to collect samples from the omasal canal Huhtanen *et al.* (1997) introduced a system that consisted of a sampling device that resided in the omasal canal and a tube that connected the device to the rumen cannula. Sample was aspirated via the sampling device into a collection vessel using a pump system that applied pulsating vacuum and pressure into the sampling device. Some modifications to the system were later suggested by Ahvenjärvi *et al.* (2001). The presence of the sampling device in the omasal canal did not affect the normal behaviour of the animals, alter digesta passage kinetics from the reticulo-rumen or the digestibility of nutrients (Huhtanen *et al.*, 1997). Comparison of omasal canal and duodenal digesta flows indicated similar N flows entering both sampling sites, whereas disappearane of fiber and minerals from the omasum and organic matter secretions into the abomasum was noted (Ahvenjärvi *et al.*, 2000). Owing to heterogenous nature of digesta flowing through the reticulo-omasal orifice the sample composition tends to be altered relative to true digesta (Huhtanen *et al.*, 1997; Ahvenjärvi *et al.* 2000, 2003). As digesta flows through the sampling device it becomes depleted of particulate matter relative to liquid. Therefore, determination of digesta flow entering the omasal canal is highly dependent on the use of reliable digesta reconstitution systems. Assessment of digesta flow measurements based on different markers indicated that no single marker is likely to results in accurate measurement of all nutrients but a combination of markers with different physical characteristics should be utilised (Ahvenjärvi *et al.*, 2003). An adequate marker system should involve markers associated with free liquid, small particulate matter and large particulate matter. According to our experience reliable measurements of ruminal feed digestibility and microbial synthesis can be attained based on the flow of digesta entering the omasal canal provided that rigorous sampling regimes are followed.

Recommendations

Flow measurements assume steady state conditions but in practise the animals exhibit daily variation and diurnal patterns in their intake. To minimize these effects we have collected twelve

digesta samples over four days to obtain a pooled sample representative of each hour between morning and evening meals. Restriction of the amount of feed offered to the animals reduces daily variation but increases diurnal variation in feed intake. The sample quantity is dependent on the number of chemical analysis required. Dry matter concentration of omasal canal digesta is typically around 34 g/kg such that twelve samples of 250 g yield approximately 100 g of DM in total. Separation of whole digesta into free liquid, small particle and large particle phases yields between 20 to 40 g DM of each phase.

Marker administration should allow marker concentrations to approach equilibrium at the sampling site before digesta collection is started. Priming doses can be used to minimize the time required for the markers to approach equilibrium. Multiple markers and digesta reconstitution technique should be used to ensure accurate flow measurements. An adequate marker system should involve markers associated with free liquid (e.g. Li or CoEDTA), small particulate matter (Yb or other rare earth metals) and large particulate matter (Cr-mordanted forage or indigestible NDF). Administration of external large particle phase marker is started 84 h prior to digesta sampling with a priming dose equal to 1.25 times the daily rate of marker administration. Following the priming dose large particle phase marker is administered twice daily into the rumen at 12-h intervals. A priming dose of liquid and small particle phase marker equal to 1.5 times the daily rate of marker administration is given 60 h prior to digesta sampling. Following the priming dose liquid and small particle phase markers are administered with a continuous infusion using a peristaltic pump.

Digesta reconstitution technique is based on the assumption that each marker results in similar digesta flow if sample is representative of true digesta. Problems with marker recoveries may violate this assumption. Therefore, marker recoveries should be routinely determined using total collection of feces over the four day collection period concurrent with digesta sampling. Digesta samples are pooled over the collection period and fractionated into liquid, small particle and large particle phases. Whole digesta is squeezed through one layer of cheesecloth. Solids retained are defined as the large particle phase. The filtrate is separated into small particle and liquid phases by centrifugation at 10 000 g for 15 min. The supernatant is defined as the liquid phase and the pellet as the small particle phase. Marker concentrations are analysed in each phase and true digesta is reconstituted either mathematically or physically as described by France and Siddons (1986). True digesta can be physically reconstituted to decrease the number of analysis or chemical composition in each phase can be analysed separately and true digesta composition can be calculated on a spreadsheet.

References

Ahvenjärvi, S., A. Vanhatalo, P. Huhtanen & T. Varvikko. (2000). Determination of reticulo-rumen and whole-stomach digestion in lactating cows by omasal canal or duodenal sampling. British Journal of Nutrition, 83, 67-77.

Ahvenjärvi, S., B. Skiba, and P. Huhtanen. (2001). Effect of heterogeneous digesta chemical composition on the accuracy of measurements of fiber flow in dairy cows. Journal of Animal Science, 79, 1611-1620.

Ahvenjärvi, S., A. Vanhatalo, K.J. Shingfield & P. Huhtanen. (2003). Determination of digesta flow entering the omasal canal of dairy cows using different marker systems. British Journal of Nutrition, 90, 41-52.

Huhtanen, P., P.G. Brotz & L.D. Satter. (1997). Omasal sampling technique for assessing fermentative digestion in the forestomach of dairy cows. Journal of Animal Science, 75, 1380-1392.
France, J. & R.C. Siddons. (1986). Determination of digesta flow by continuous marker infusion. Journal of Theoretical Biology, 121, 105-119.

Discussion summary

Discussion followed on the procedures, problems and recommendations. This is a relatively new method to measure digesta flow and was developed from work by Huhtanen and Satter. The advantage is that the more robust rumen cannula can be used, greatly prolonging the experimental life of the animal with minimal maintenance compared to abomasal or intestinal cannulae. The same procedures and precautions need to be taken as were outlined by Faichney during the 1974 ISRP. The advantage comes in the fistulation site and sampling procedure rather than any methodological advantages.

The tip of the sampling tube is placed into the omasal canal by hand via the rumen fistula and after locating the omasal orifice. A weight is used to position the sampling tube within the omasal canal with the weight maintained within the abomasum. It is positioned about 2d prior to sampling and before sampling the tip position is checked again. Sampling occurs under suction and a recommendation for the procedure is given above. A 3 phase marker system is used and there is some discrimination against larger particles if care is not taken. Hence it is important to have a marker associated with large particles. CrEDTA or CoEDTA is used for the liquid phase, Yb is used for the small particle phase and large particle-indigestible NDF (LP-INDF) is used for the large particle phase. Digesta reconstitution occurs according to the principles outlined by Faichney. INDF is measured using 17u pore size nylon bags incubated within the rumen for 14d. Recoveries of INDF have been checked by this procedure and found to approximate 100%. A question arose into the establishment of a direct fistula into the omasum but the technical difficulties were immense. The current procedure is much simpler. Animals required a period of adaptation and odd animals showed a depression in intake but for the most part no problems were experienced with animals. Feed type has been largely chopped maize silage and long hay may cause problems with the maintenance of the position of the tube within the omasal canal. There was no effect of position of tube within the omasal canal on the particle size of the faeces, further supporting the idea that the size of the omasal orifice has no influence on the size of particles which escape the rumen and a self sieving mechanism of digesta is a more appropriate model. The sampling regime usually involved 3 samples/day, with a 4d sampling regime after a preliminary infusion procedure of 4d to reach plateau concentration of markers.

Index

C

D

N

O

P

R

S

T

U

V

W

X

Z