The Pentose Phosphate Pathway

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Preface

In 1964, the English edition of "Non-Glycolytic Pathways of Metabolism of Glucose" by Hollmann and Touster was published by Academic Press. A substantial part of the book was devoted to the pentose phosphate cycle and the practical techniques used in its investigation. Over the intervening 20 years or so, a considerable additional corpus of knowledge about the pathway has been acquired. It is my purpose to document and review this knowledge in a form that will be helpful to research workers in the field, to students, and to those doctors and scientists who require more information than may be found in the standard textbooks.

As a general rule, work carried out prior to 1960 has been ignored, since these early developments have been adequately described in the Hollmann and Touster volume. However, in Chapter 3, where the preparation, properties, and analysis of intermediates of the pathway are discussed, it has, in some cases, been necessary to go back to work carried out in the 1930s.

The properties of the enzymes of the pathway have been reviewed in the two series entitled "The Enzymes," one series edited by Boyer, Lardy, and Myrbäck (1961–1963) and the other by Boyer (1972), and appropriate references are given in Chapter 4 of this volume. A vast amount of information is available on glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and the reader is referred to other sources for detailed information about these two enzymes. The remaining enzymes of the pathway are discussed in Chapter 4, with references to "The Enzymes" for supplementing details.

Professor Bernard Landau graciously agreed to write a chapter on the use of radioisotopes in investigating the pathway, and his contribution in Chapter 12 is a most welcome description of this important topic.

The pentose phosphate pathway described in these pages is that found in animals and microorganisms, and no attempt has been made to discuss the reductive pentose phosphate pathway of photosynthesis, for which the reader is referred to works dealing specifically with that subject.

I wish to record my gratitude to Professor H. L. Kornberg for making available to me the Biochemistry Department library and other libraries at

the University of Cambridge and to Professor Peter Walker for reading and checking Chapter 10. My thanks are due also to the University of Zimbabwe for sabbatical leave, during which much of this book was written, and to my wife, Catherine, for her continued help and encouragement.

Terry Wood

List of Abbreviations

AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
Ara	arabinose
Ara-5-P	arabinose 5-phosphate
CD	circular dichroism
*p*CMB	*para*-chlormercuribenzoic acid
DHA	dihydroxyacetone
DHAP	dihydroxyacetone phosphate
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
EDTA	ethylenediamine tetraacetic acid
Epimerase	D-ribulose-5-phospate 3-epimerase
Er-4-P	erythrose 4-phosphate
Er-4-homo-P	erythrose 4-homophosphonate
Fru	fructose
F-6-P	fructose 6-phosphate
F-1,6-PP	fructose 1,6-bisphosphate
GAP	glyceraldehyde 3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-6-P	glucose 6-phosphate
G-6-PDH	glucose-6-phosphate dehydrogenase
GIP	*sn*-glycero-3-phosphate
GIPDH	*sn*-glycero-3-phosphate dehydrogenase
GSH	reduced glutathione
GSSG	oxidized glutathione
Hb	hemoglobin
HPLC	high-pressure liquid chromatography
Isomerase	D-ribose-5-phosphate ketol-isomerase
K_i	inhibition constant
K_m	Michaelis constant
LDH	lactate dehydrogenase
NAD	nicotinamide adenine dinucleotide

NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
Oct	octulose
ORD	optical rotatory dispersion
P_i	inorganic phosphate
PFK	phosphofructokinase
6-PG	D-6-phosphogluconate
6-PGDH	D-6-phosphogluconate dehydrogenase
3-PGA	D-3-phosphoglyceric acid
1,3-PPGA	D-1,3-bisphosphoglyceric acid
PGI	phosphoglucoisomerase
PGK	3-phosphoglycerate kinase
pI	isoelectric point
PRPP	phosphoribosyl pyrophosphate
R-2-P	ribose 2-phosphate
R-3-P	ribose 3-phosphate
R-4-P	ribose 4-phosphate
R-5-P	ribose 5-phosphate
Rib	ribose
RT	room temperature
Ru	ribulose
Ru-5-P	ribulose 5-phosphate
Ru-1,5-PP	ribulose 1,5-bisphosphate
SBPase	sedoheptulose bisphosphatase
S-7-P	sedoheptulose 7-phosphate
S-1,7-PP	sedoheptulose 1,7-bisphosphate
TA	transaldolase
TK	transketolase
TPI	triose-phosphate isomerase
TPP	thiamine pyrophosphate
Triose-P	triose phosphate
U	international unit (1 micromole/minute)
Xu	xylulose
Xu-5-P	xylulose 5-phosphate
Xu-1,5-PP	xylulose 1,5-bisphosphate
Xyl	xylose

All the sugars and sugar phosphates in the above list are of the D-configuration. Some of the abbreviations for sugars differ from those recommended by the International Union of Biochemistry [see *Biochem. J.* **171,** 1–19 (1978)].

1

Introduction

INTRODUCTION

The oxidation of glucose 6-phosphate to 6-phosphogluconate in the presence of the coenzyme NADP was established nearly 50 years ago by Warburg and Christian (1936, 1937), and it was later recognized that further oxidation to unknown products could occur. The research of Dickens (1938a,b) led to the identification of ribose 5-phosphate as one of the oxidation products. Finally, the combined efforts of two principal groups of American research workers, headed by Bernard Horecker and Efraim Racker, respectively, led to the description of the pentose phosphate pathway in the form it is usually depicted today.

Reviews of knowledge about the pathway then current may be found in the articles by Horecker and Mehler (1955), Korkes (1956), Pon (1964), Sable (1966), Axelrod (1967), Horecker (1968), and Pontremoli and Grazi (1969) and in the chapter on the pentose phosphate cycle in the monograph "Non-Glycolytic Pathways of Metabolism of Glucose" by Hollmann and Touster (1964). Accounts of the historical development of the subject have been provided by Horecker (1976) and by Florkin and Stotz (1979), while

certain specialized aspects of the pathway, its relationship to glycolysis and its regulation in rat liver, have been treated by Severin and Stepanova (1981) and Krebs and Eggleston (1974), respectively.

Following the elucidation of the pathway in the mid-1950s, interest in it lapsed for a while but gradually revived as more and more of the enzymes of the pathway and the intermediates became commercially available. The experimental exploration of problems concerning the pathway and the properties of its enzymes thus became a practical proposition. The renewed interest has been particularly focused on the enzymes of the pathway, on the function of the pathway in such tissues as brain, lung, and heart, and on the relationship between the pathway and such processes as proline synthesis, phagocytosis, ribose synthesis, and the formation of bacterial cell wall constituents.

The basic formulation of the pathway has been called into question by Williams and his co-workers, and the matter is, at present, a subject of active research. Williams's ideas, along with those of others, are discussed in Chapters 2 and 12.

The chemistry, properties, and analysis of the intermediates of the pathway are presented in Chapter 3, and their occurrence in living matter, in Chapter 5. Similarly, for enzymes, their basic properties are described in Chapter 4 and their distribution in nature in Chapter 6, while information about the properties of the two dehydrogenases of the pathway may be found in the articles by Noltmann and Kuby (1963) and by Pontremoli and Grazi (1969). Chapters 7, 8, and 9 describe the operation and control of the pathway in broken cells and intact tissues, while some aspects of medical interest are considered in Chapters 10 and 11. Finally, in Chapter 12, radioisotope techniques and the results obtained with them are discussed.

2

Formulation of the Pathway

INTRODUCTION

It is generally recognized that the pentose phosphate pathway fulfills two main functions in metabolism, the formation of ribose 5-phosphate for nucleotide and nucleic acid synthesis and the generation of NADPH as a source of reducing equivalents for biosynthetic reactions. In addition, other intermediates of the pathway may be required in biosynthesis, for example, erythrose 4-phosphate is needed by certain microorganisms for the manufacture of aromatic amino acids (Srinivasan *et al.*, 1955) and sedoheptulose 7-phosphate for incorporation into cell wall glycolipids (Eidels and Osborn, 1971).

The pathway was formulated in its classical form as a cycle by Horecker and Mehler (1955) from their knowledge of the enzymes and intermediates of the pathway and from the results of experiments in which [^{14}C]ribose 5-phosphate was incubated with extracts of rat liver, pea root, and pea leaf (Gibbs and Horecker, 1954; Horecker *et al.*, 1954). From the characteristic labeling pattern of the hexose phosphate and triose phosphate products it was deduced that the pathway consisted of the sequence of reactions shown

in Fig. 2.1, in which six molecules of hexose phosphate traverse the cycle with the formation of six molecules of carbon dioxide and six molecules of pentose phosphate. These six molecules of pentose phosphate are converted into four molecules of hexose phosphate and two molecules of triose phosphate. The two triose phosphate molecules may be condensed together

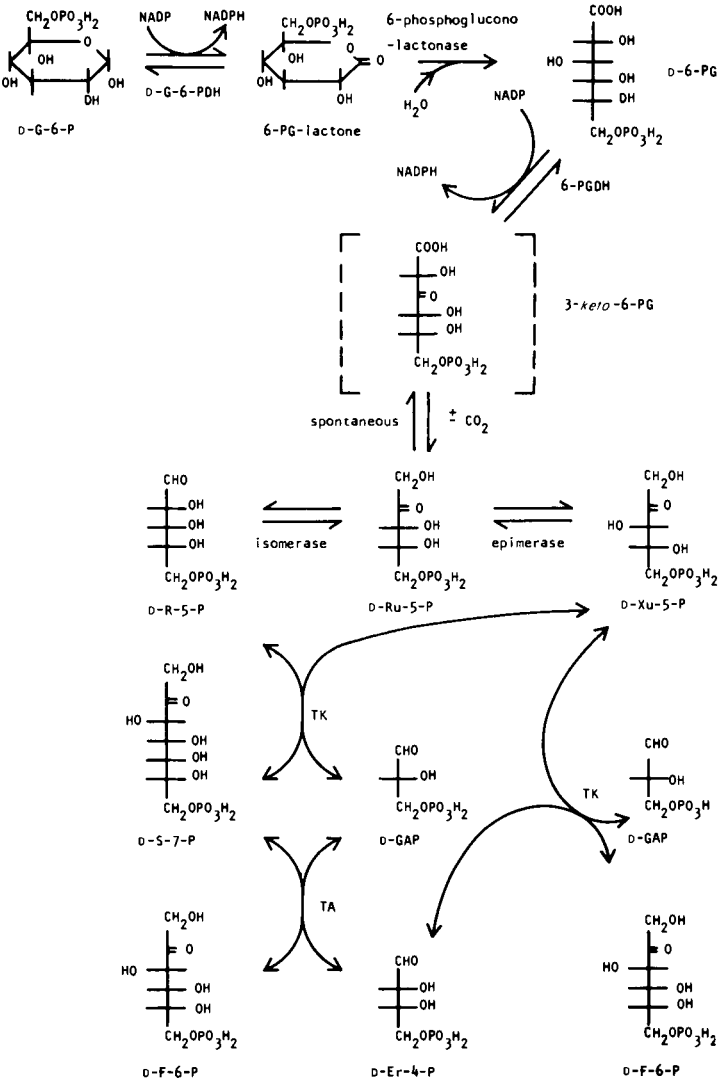


Fig. 2.1. The classical formulation of the pentose phosphate pathway (Horecker and Mehler, 1955).

by aldolase to form hexose bisphosphate, which can be split by a phosphatase, forming another molecule of hexose monophosphate and a molecule of inorganic phosphate. Thus, the six molecules of hexose phosphate entering the cycle emerge as five molecules of hexose phosphate reentering the glycolysis pathway and six molecules of carbon dioxide derived exclusively from C-1 of glucose 6-phosphate.

Although originally presented as the "pentose phosphate cycle," it has gradually become clear that the "cycle" can operate as two separate pathways, the "oxidative branch" and the "nonoxidative branch," and the name "pentose phosphate pathway" now seems more appropriate. The oxidative branch generates pentose phosphate and NADPH via the two dehydrogenases at the start of the pathway. The nonoxidative branch equilibrates the pentose phosphates with the hexose phosphates of glycolysis via the remaining pathway enzymes, in particular transketolase (TK) and transaldolase (TA). Pentose phosphate formed by the oxidative branch and not further utilized is converted back to hexose phosphate by the nonoxidative branch and reenters the glycolysis pathway.

EXPERIMENTS WITH ISOTOPES

The "classical" pathway, as formulated by Horecker and Mehler (Fig. 2.1), leads to the redistribution of the carbon atoms of hexose phosphate illustrated in Fig. 2.2. If [2-^{14}C]glucose is administered, then the isotope will appear in C-1 of pentose phosphate and will have a characteristic distribution in C-1, C-2, and C-3 of the hexose phosphate formed by the cycle and reentering the glycolysis pathway. When the hexose 6-phosphate is isolated from the tissue and the radioactivity in each individual carbon atom is determined, the labeling pattern and the ratios of the specific activities of the individual carbon atoms may be used to calculate the "percentage pentose cycle." This latter quantity has been defined as the percentage of glucose entering the tissue that is metabolized to carbon dioxide and triose phosphate by the pentose phosphate cycle (Katz and Wood, 1960; Wood *et al.*, 1963). Other ways of calculating the contribution of the cycle have been used and are discussed by Wood *et al.*, (1963).

Until 1958, workers attempting to calculate the contribution of the pentose cycle with the aid of radioisotopes considered that the hexose phosphate produced by the cycle was converted immediately to hexose bisphosphate and further metabolized. Dawes and Holms (1958, 1959) were the first to consider that the fructose 6-phosphate formed by the cycle could be isomerized to glucose 6-phosphate and enter the cycle again. This "recycling," as it was called, was taken into account by Katz and Wood (1960)

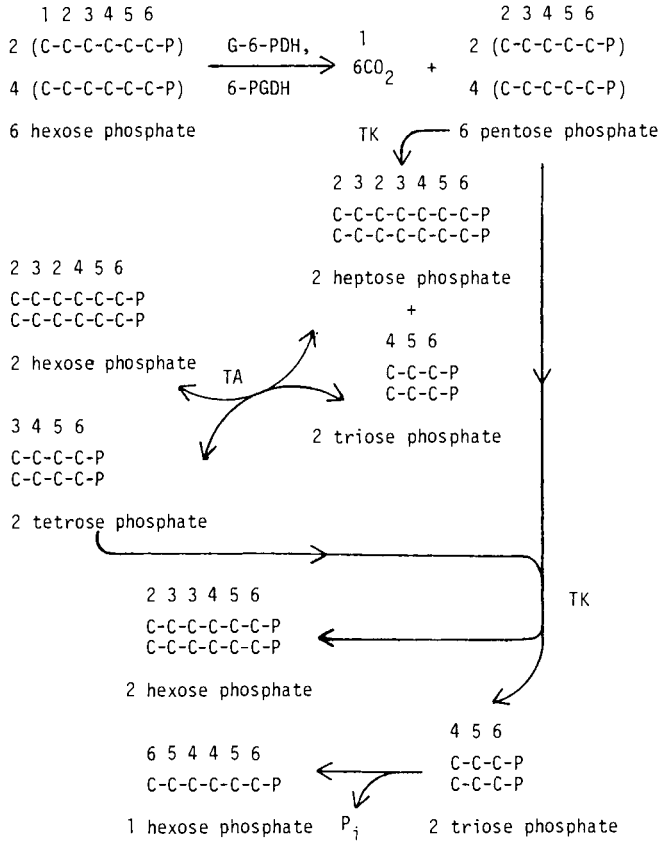


Fig. 2.2. Distribution of carbon atoms after passage of six molecules of hexose phosphate through the classical pathway (Axelrod, 1967).

when they described their method for calculating the percentage pentose cycle from the ratios of the specific activities of the first three carbon atoms of the hexose phosphate pool when either [2-^{14}C]glucose or [3-^{14}C]glucose was used as substrate.

The method of Katz and Wood (1960), as originally conceived, failed to explain how radioisotope could arrive in C-4, C-5, and C-6 of the hexose phosphate when [2-^{14}C]glucose or [3-^{14}C]glucose was metabolized. The original scheme was then modified to take into account incomplete equilibration of glucose 6-phosphate and fructose 6-phosphate (Landau *et al.*, 1964), the effect of exchange reactions catalyzed by transaldolase (Ljungdahl *et al.*, 1961; Landau and Katz, 1964; Landau and Bartsch, 1966; Katz and Rognstad, 1967; Katz *et al.*, 1975) and transketolase (Katz and Rogn-

stad, 1967; Clark *et al.*, 1971), incomplete equilibration of glyceraldehyde phosphate (GAP) and dihydroxyacetone phosphate (DHAP) (Katz *et al.*, 1966), and incomplete equilibration of the pentose phosphates in the tissue (Katz and Rognstad, 1967).

In 1971, Williams *et al.*, published the results of their study of the metabolism of [2-^{14}C]glucose by rabbit liver. They isolated glucose 6-phosphate from the tissue and found a high proportion of the isotope in C-6. This distribution could not be explained by the pentose cycle or by any other known pathway of glucose metabolism. Further experiments were performed with rat liver, and the original experiments of Horecker and Gibbs were repeated (Williams *et al.*, 1978a,b). Although the experiments with an extract of rat liver acetone powder yielded results akin to those of Horecker *et al.* (1954) after 17-hr incubation, at shorter incubation times heavy labeling of C-6 was observed, and considerable quantities of isotope were present in C-4. It was decided that these results could only be explained if the pathway contained intermediates and enzymes other than those included in the "classical" pathway. Evidence for the presence of arabinose 5-phosphate, octulose (OCT) phosphates, and additional enzymes was obtained, and an enlarged "liver-type" or "L-type" pathway was presented (Fig. 2.3) (Williams and Clark, 1971; Williams *et al.*, 1978a). For an account of these developments and a critical review of the work leading to the formulation of the classical pathway the reader is referred to papers by Williams and co-workers (Williams and Clark, 1971; Williams *et al.*, 1978a,b; Williams, 1980) and a review by Mujaji (1980).

Longenecker and Williams (1980a) have described how [2-^{14}C]glucose and [5-^{14}C]glucose may be used to estimate the contribution of the L-type cycle to glucose metabolism. In isolated rat liver hepatocytes, they calculated that between 22 and 30% of the glucose metabolized was catabolized by the pathway (Longenecker and Williams, 1980b), and in rat epididymal fat tissue, 42% of the glucose in a starved animal and 60% in a starved–refed animal went by the pathway (Blackmore *et al.*, 1982). In hepatocytes, they concluded, the L-type pathway operated exclusively, while in fat tissue, the reactions followed the classical scheme or "F-type" pathway, although some elements of the L-type pathway appeared to be present.

THE L-TYPE PATHWAY

The L-type pathway (Fig. 2.3) is distinguished from the classical or F-type pathway by four additional intermediates and three additional enzymes. It ascribes no role to transaldolase, although the activity of this enzyme is heavily relied on to explain the incorporation of label into C-4, C-5, and C-6

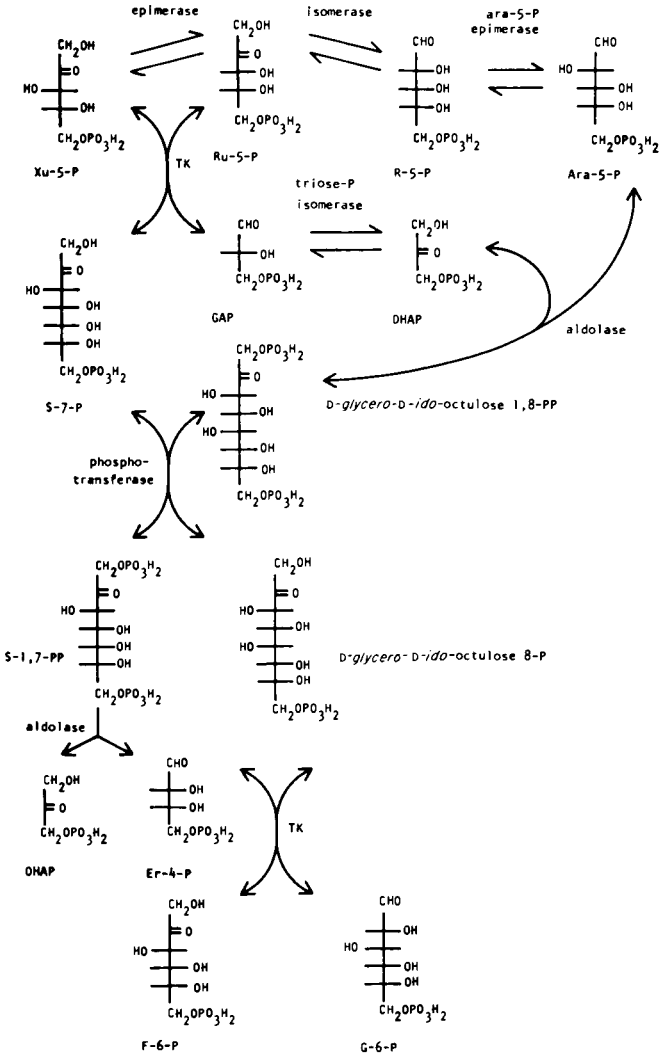


Fig. 2.3. The L-type pathway. (From Williams *et al.*, 1978a. Reprinted by permission from *Biochem. J.* 176, 257–282. Copyright © 1978. The Biochemical Society, London.)

of the hexose phosphate pool (Williams and Clark, 1971; Williams *et al.*, 1978a; Williams, 1980; Longenecker and Williams, 1980a). The new intermediates are D-arabinose 5-phosphate, D-*glycero*-D-*ido*-octulose 1,8-bisphosphate, D-*altro*-heptulose 1,7-bisphosphate, and D-*glycero*-D-*ido*-octulose 8-phosphate. The pathway also invokes the action of aldolase, a D-arabinose 5-phosphate 2-epimerase, and a phosphotransferase catalyz-

ing the transfer of a phosphate group between heptulose and octulose bisphosphates.

The evidence for this pathway rests partly on the identification by paper chromatography (Williams *et al.*, 1978a) and gas–liquid chromatography (Blackmore *et al.*, 1982; Williams and Blackmore, 1983) of arabinose among the free sugars obtained by acid phosphatase treatment of the reaction products. Further evidence for arabinose 5-phosphate as an intermediate was its rapid utilization by enzymes of the pathway (Williams *et al.*, 1978a).

The interconversion of ribose 5-phosphate and arabinose 5-phosphate was investigated by Wood and Gascon (1980), who were unable to find any evidence of interconversion by rat muscle or rat liver supernatants or extracts of a rat liver acetone powder. Moreover, no appreciable utilization of arabinose 5-phosphate in the presence of an extract of rat liver acetone powder could be detected by paper chromatography or by spectrophotometry, as reported by Williams *et al.* (1978a). In effect, arabinose 5-phosphate appeared to be an inhibitor of the pathway rather than a substrate (Wood and Gascon, 1980; Wood, 1981b).

It is noteworthy that ribulose 5-phosphate was completely and conspicuously absent from the list of products formed from ribose 5-phosphate in the experiments of Williams *et al.* (1978a), whereas an amount of arabinose 5-phosphate was found equivalent to the ribulose 5-phosphate that would have been expected. In some similar experiments in which ribose 5-phosphate was incubated with rat liver enzymes, ribulose 5-phosphate accumulated in parallel with xylulose 5-phosphate and in comparable amount (Wood, 1974c). In view of the pH value of 8 that was used in neutralization of the extracts, it is possible that the arabinose 5-phosphate was a transformation product of ribulose 5-phosphate. The presence of arabinose 5-phosphate among the products of 6-phosphogluconate decarboxylation was reported by Scott and Cohen (1951) but later was shown to be an artifact formed by alkaline isomerization of ribulose 5-phosphate to a mixture of ribose and arabinose 5-phosphates (Cohen, 1953; Sable, 1966).

Williams's group (Blackmore *et al.*, 1982) has reported small amounts of arabinose 5-phosphate among the intermediates formed by the F-type pathway in epididymal tissue, and the group suggested that it may have been formed by enzymic epimerization of ribose 5-phosphate or by the combined action of transketolase and transaldolase [Eqs. (1) and (2)].

$$\text{D-glucose 6-phosphate} + \text{D-fructose 6-phosphate} \overset{\text{TK}}{\rightleftharpoons} \text{D-}\textit{glycero}\text{-D-}\textit{ido}\text{-octulose 8-phosphate} + \text{D-erythrose 4-phosphate} \tag{1}$$

$$\text{D-}\textit{glycero}\text{-D-}\textit{ido}\text{-octulose 8-phosphate} + \text{D-erythrose 4-phosphate} \overset{\text{TA}}{\rightleftharpoons} \text{D-}\textit{altro}\text{-heptulose 7-phosphate} + \text{D-arabinose 5-phosphate} \tag{2}$$

Evidence for the existence of the other intermediates of the L-type pathway is much stronger. Vanderheiden (1964) and Bartlett and Bucolo (1968) reported heptulose and octulose bisphosphates in erythrocyte extracts, and Paoletti *et al.*, (1979b) determined D-sedoheptulose 7-phosphate, D-*glycero*-D-*ido*-octulose 8-phosphate, D-*glycero*-D-*altro*-octulose 8-phosphate, and the corresponding bisphosphates in rat liver by column chromatography and enzymatic assay. The phosphotransferase has been assayed in rat epididymal fat tissue and found to catalyze the reaction in the direction of *altro*-heptulose 1,7-bisphosphate formation at a rate three times the overall rate of hexose phosphate formation from ribose 5-phosphate (Blackmore *et al.*, 1982).

At the time of writing, the evidence for the existence of the L-type pathway appears somewhat tenuous (for a discussion, see Katz, 1981; Landau, 1981; Wood, 1981b; Williams, 1981; Landau and Wood, 1983a,b; Williams *et al.*, 1983). Neither of the two new enzymes of the pathway has been purified and characterized, although the Williams group (Blackmore *et al.*, 1982) has reported the partial purification of a D-arabinose 5-phosphate isomerase from rat liver. It is possible that, apart from arabinose 5-phosphate, which was reported to accumulate in considerable amount (Williams *et al.*, 1978a), the other new seven- and eight-carbon intermediates represent the products of side reactions catalyzed by the main enzymes of the pathway.

Using a glucose 6-phosphate isomerase-deficient strain of cultured Chinese Hamster cells, Morgan (1981) has shown that these cells can form glucose 6-phosphate from ribose 5-phosphate only when the missing enzyme is added. This result disproves the operation in these cells of both the L-type pathway and that suggested by Severin and Stepanova (1981), in which glucose 6-phosphate is formed directly from intermediates of the respective pathways.

On the basis of experiments with labeled xylitol and xylulose it has been denied that the L-type pathway operates in liver cells, and it was concluded that labeling patterns were in accordance with the classical scheme (Rognstad and Katz, 1974; Rognstad *et al.*, 1982).

Williams's group has published data and a radioautogram showing labeling of hexose and pentulose phosphates and a heptose phosphate when [U-^{14}C]D-arabinose 5-phosphate was incubated with a liver enzyme preparation and a fivefold molecular concentration of unlabeled D-ribose 5-phosphate.

The yield of glucose 6-phosphate in this experiment was small compared with the yield in the absence of arabinose 5-phosphate. This was attributed to the inhibitory properties of the latter. When the aldolase in the preparation was removed by reaction with Sepharose-bound liver aldolase antibody, hexose 6-phosphate formation was prevented but could be restored

by the addition of liver aldolase, but not by the addition of muscle aldolase (Bleakley *et al.*, 1984).

Although these findings are of considerable interest, it is not evident that the isotope experiments prove more than the ability of the enzyme preparation to catalyze exchange reactions that incorporate arabinose 5-phosphate into intermediates of the pathway. No reason was given why the conversion of arabinose 5-phosphate into these intermediates could not be shown directly without the use of radioisotopes; yet, in earlier experiments, this conversion was claimed to have been demonstrated (Williams *et al.*, 1978a).

If the existence of the L-type pathway is to be unequivocally established, the use of radioisotopes should be eschewed, the new enzymes of the pathway purified and their properties determined, and the interconversion of the intermediates demonstrated by unambiguous chemical methods.

OTHER ALTERNATIVE FORMULATIONS OF THE PATHWAY

The formation of pentose phosphate from hexose phosphate and triose phosphate by the nonoxidative branch of the pathway was considered by Sable (1966). The scheme he put forward (Fig. 2.4) included a step contained in an earlier formulation by Couri and Racker (1959) by which triose phosphate was condensed with erythrose 4-phosphate in the presence of aldolase to form sedoheptulose 1,7-bisphosphate, which was then hydrolyzed by a specific sedoheptulose 1,7-bisphosphatase (SBPase) to form the monophosphate. The latter compound could then condense with a second molecule of triose phosphate in a transketolase reaction to form two molecules of pentose phosphate. Transaldolase did not participate in the scheme, and the overall labeling pattern of the products would have been the same as with reversal of the nonoxidative branch of the classical scheme.

The previous scheme is almost identical to two formulations proposed by Blackmore and Shuman (1982) and by Susskind *et al.* (1982). In the former, suggested to apply to rat liver, the phosphatase activity is reversed by the action of phosphofructokinase (PFK), and both enzymes are under the control of fructose 2,6-bisphosphate and hormones. In the latter formulation, which describes the pathway in *Entamoeba histolytica*, the interconversion of the seven-carbon mono- and bisphosphates is brought about reversibly by the pyrophosphate-dependent phosphofructokinase present in that organism.

The formation of hexose phosphate from ribose 5-phosphate by the nonoxidative branch of the pathway was investigated using the supernatant fraction from various tissues (Wood, 1974c). A marked difference was found between liver and whole uterus on the one hand and muscle on the other. With liver and uterus, hexose monophosphate was formed, and the

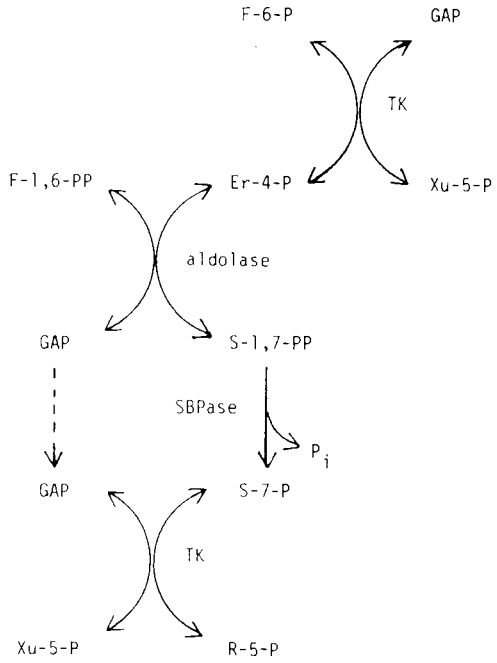


Fig. 2.4. An alternative nonoxidative branch. (From Sable, *Adv. Enzymol. Relat. Subj. Biochem.* Copyright © 1966 by John Wiley & Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.)

usual intermediates accumulated, including appreciable amounts of triose phosphate. Muscle supernatant fractions produced negligible amounts of hexose and triose phosphates but accumulated *sn*-glycerol-3-phosphate (GlP).This divergence in behavior was apparently due to the reduction of the triose phosphate intermediates by muscle enzymes. The formation of fructose 6-phosphate from ribose 5-phosphate by liver and uterus supernatants could be followed in the spectrophotometer at 340 nm if NADP, phosphoglucose isomerase (PGI), glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase were added to the system. When muscle supernatant was used, no NADP reduction was observed. This observation was explained by the presence of an NADP–glyceraldehyde phosphate oxidoreductase (EC 1.1.1.177; Anonymous, 1980) (Wood, 1973a; Glushankov *et al.*, 1976) that reoxidized the NADPH formed by dehydrogenation of the hexose phosphate as rapidly as it appeared (Wood, 1974c).

A function for the newly discovered enzyme was envisaged as a participant in the pathway outlined in Fig. 2.5. In this pathway, transaldolase,

which is low in rat muscle (Tan and Wood, 1969; Severin and Stepanova, 1981) plays no part, and triose phosphate, generated from pentose phosphate by the sequential action of transketolase, phosphofructokinase, and aldolase, is used to reoxidize NADPH formed by the oxidative branch. In this way pentose phosphate formation for nucleotide synthesis is facilitated in the absence of NADPH turnover by other means.

Another formulation of the nonoxidative branch has been proposed by Severin and Stepanova (1981), which places erythrose 4-phosphate in a central role, able to form pentose phosphate and to be formed from hexose phosphates by the transketolase reaction (Fig. 2.6). This pathway was intended to explain how pentose phosphates were formed from hexose phosphates by heart muscle extracts in which transaldolase was low or absent. Although evidence was provided that erythrose 4-phosphate could be synthesized from glucose 6-phosphate and fructose 6-phosphate by heart muscle enzymes, it may be questioned whether this synthesis is the result of transketolase action and whether it would have physiological significance in the light of reports that glucose 6-phosphate is a very poor acceptor in the transketolase reaction (Pontremoli *et al.*, 1960; Wood and Gascon, 1980; Wood, 1981a; Paoletti, 1983).

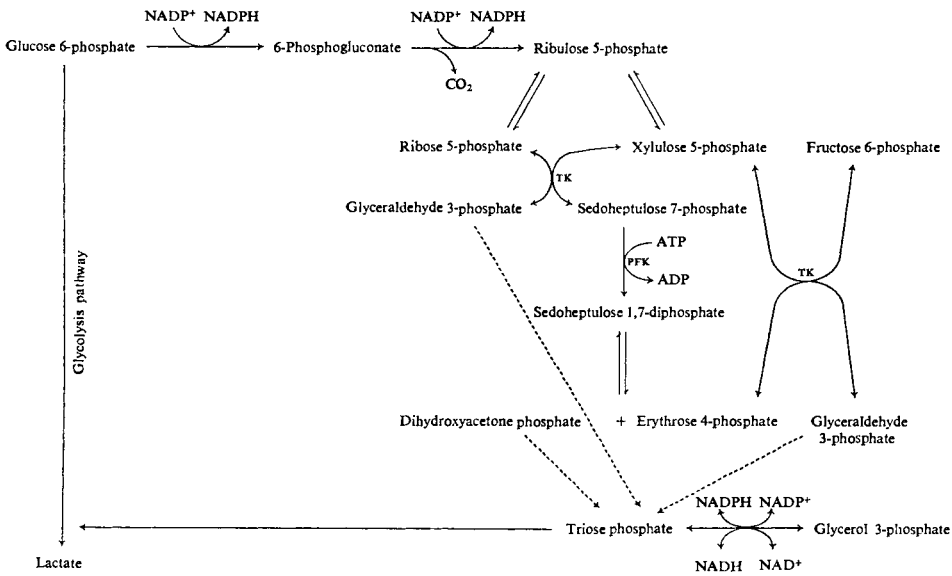


Fig. 2.5. Alternative formulation of the pathway in muscle. (From Wood, 1974c. Reprinted by permission from *Biochem. J.* **138**, pp. 71–76. Copyright © 1974. The Biochemical Society, London.)

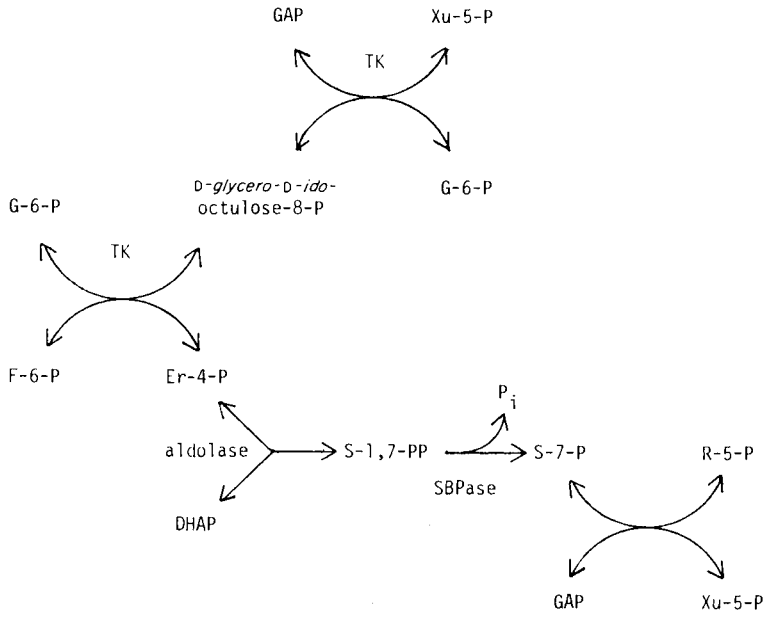


Fig. 2.6. An alternative nonoxidative branch. (Reprinted with permission from Adv. Enz. Reg. **19,** Severin and Stepanova, Interrelationship between glycolysis and the anaerobic part of the pentose phosphate pathway of carbohydrate metabolism in the myocardium. Copyright 1981, Pergamon Press Ltd.)

D-ERYTHROSE 4-PHOSPHATE AS AN INTERMEDIATE OF THE PATHWAY

Following its synthesis by Ballou *et al.*, (1955) and the demonstration that it served as a substrate for transaldolase (Kornberg and Racker, 1955), erythrose 4-phosphate was incorporated into the classical pentose phosphate pathway. It has been questioned whether this intermediate has ever been detected in living tissues (Williams *et al.*, 1980). Criticism of reports of the presence of this compound center around the doubtful specificity of the assay methods used. When more specific assays were applied, it was not detectable in liver, adipose tissue, or systems known to accumulate intermediates of the pathway (Paoletti *et al.*, 1979c).

A number of possible reasons were advanced for the failure to detect erythrose 4-phosphate in tissues. The most plausible one is its ready reactivity with transketolase, transaldolase, and aldolase. The Michaelis constants of these enzymes for erythrose 4-phosphate are very low and the rates of reaction high compared to some other substrates (Novello and McLean, 1968; Severin and Stepanova, 1981; Chapter 4). It has also been suggested

that erythrose 4-phosphate is "stored" as sedoheptulose 1,7-bisphosphate by reaction with dihydroxyacetone phosphate and aldolase and that very little free tetrose phosphate is present in tissues (Horecker and Mehler, 1955). A possibility not specifically considered by Williams *et al.* (1980) is that erythrose 4-phosphate under physiological conditions never appears in the free state but is formed and metabolized only via the tightly coupled transketolase–transaldolase reaction described in the next section.

THE TRANSKETOLASE–TRANSALDOLASE COUPLED REACTION AND THE EXISTENCE OF ENZYME COMPLEXES

The reactions of the pathway have been generally regarded as being catalyzed by a collection of soluble enzymes acting independently of each other, although Baquer and McLean (1972a) have demonstrated that part of the activity in some rat tissues is associated with a particulate fraction. It was reported by Bonsignore and co-workers (Bonsignore *et al.*, 1957, 1958) that thoroughly dialyzed liver extracts could synthesize sedoheptulose 7-phosphate and xylulose 5-phosphate directly from fructose 6-phosphate in the absence of any other added intermediates. Further experiments confirmed that the reaction could be catalyzed by appropriate mixtures of highly purified samples of the two enzymes (Pontremoli *et al.*, 1960, 1961b), and the mechanism of the process was suggested to be the reaction of triose and tetrose phosphate released at the active site of one enzyme, with the corresponding fragments bound to the active site of the other [Eqs. (3)–(7)]. In this way there was no requirement for added triose or tetrose phosphate and no release of these intermediates inside the cell (Bonsignore *et al.*, 1957, 1958, 1962; Horecker *et al.*, 1963).

$$\text{D-F-6-P} + \text{TA} \rightleftharpoons \text{D-GAP} + \text{dihydroxyacetone-TA} \quad (3)$$

$$\text{D-F-6-P} + \text{TK} \rightleftharpoons \text{D-Er-4-P} + \text{glycolaldehyde-TK} \quad (4)$$

$$\text{dihydroxyacetone-TA} + \text{D-Er-4-P} \rightleftharpoons \text{D-S-7-P} + \text{TA} \quad (5)$$

$$\text{glycolaldehyde-TK} + \text{D-GAP} \rightleftharpoons \text{D-Xu-5-P} + \text{TK} \quad (6)$$

Overall,

$$2\text{D-F-6-P} \rightleftharpoons \text{D-S-7-P} + \text{D-Xu-5-P} \quad (7)$$

Clearly, if the previous mechanism were of general significance, it would account for the inability of many investigators to detect appreciable amounts of erythrose 4-phosphate in metabolizing systems and would markedly alter the formulation of the nonoxidative branch of the pathway. It was reported that sedoheptulose 7-phosphate could serve as a substrate

for the coupled reaction to yield fructose 6-phosphate and D-*glycero*-D-*altro*-octulose 8-phosphate (Williams and Blackmore, 1983).

Although it was not proposed that the coupling of transketolase and transaldolase depended on the existence of a complex of the two enzymes, such a configuration would appear logical. Complex formation between the two enzymes from *Candida utilis* has been reported by Kiely, *et al.*, (1969) and Wood (1981a), and the existence of a tight complex of transketolase and glyceraldehyde phosphate dehydrogenase (GAPDH) is well documented (Kochetov *et al.*, 1970a; Kochetov and Chernov, 1970, 1971; Heinrich and Wiss, 1971a; Saitou *et al.*, 1974; Wood and Muzariri, 1981). There is some evidence also for a termolecular complex of transketolase, transaldolase, and glyceraldehyde phosphate dehydrogenase (GAPDH) (Wood *et al.*, 1985).

CONCLUSION

It is clear from the foregoing that the formulation of the pathway is being reconsidered in several quarters in order to accommodate new facts. The use of radioisotopes to study the functioning of the pathway might be regarded as having raised more problems than it has solved. However, whatever the final form of the pathway, or pathways, it should be capable of describing the distribution of isotope found in the intermediates when a specifically labeled substrate is supplied and should be compatible with the known specificities and kinetic properties of the constituent enzymes.

3

Preparation, Properties, and Analysis of the Intermediates of the Pathway

INTRODUCTION

Apart from 6-phosphogluconic acid, all the intermediates of the pathway are phosphorylated sugars that differ from each other only according to (1) whether there are one or two phosphate residues in the molecule; (2) the nature of the sugar; and (3) its linkage to the phosphate group.

An important characteristic is the alkali lability of the ketopentoses and their phosphates and their ability to react in the cysteine – carbazole reaction to give an intense red-purple color. Sedoheptulose and its 7-phosphate also give characteristic color reactions, a delicate rose with cysteine – sulfuric acid and a blue-green color with orcinol and ferric chloride (Dische, 1953; Sonka and Kucharova, 1956).

The intermediates may be prepared from each other by using the enzymes of the pathway or by phosphorylation of the free sugars, either

chemically or by using specific kinases. Other methods involve the condensation of smaller fragments with aldolase and straight chemical synthesis (Serianni *et al.*, 1979, 1982).

General information and analytical techniques may be found in the monographs by Hollmann and Touster (1964) and Pigman (1957) and in the treatise edited by Fasman (1975). The articles by Ashwell (1957, 1966) and Benson (1957) in "Methods in Enzymology" and those in Bergmeyer (1974) may also be consulted.

GENERAL ANALYTICAL METHODS

The Cysteine–Carbazole Reaction

The cysteine–carbazole reaction was introduced by Dische and Borenfreund (1951) as a general colorimetric method for ketosugars and trioses. It was soon recognized that the reaction was particularly sensitive to small amounts of ribulose, which gave four times as much color in 15 min as an equimolecular amount of fructose, and the same amount of color from both sugars was only obtained after fructose had reacted at room temperature for 19 hr (Cohen, 1953). It was also reported that aldopentoses and ribulose 5-phosphate were less reactive than ribulose (Cohen, 1953) and that, whereas ribulose developed its maximal color after 15 min at 37°C, xylulose required 60 min and xylulose 1-phosphate 20 hr for maximal color development. However, equimolecular amounts of xylulose 1-phosphate and free xylulose yielded the same maximal absorbance (Lampen, 1953).

Axelrod and Jang (1954) used the cysteine–carbazole reaction to follow D-ribulose 5-phosphate formation from D-ribose 5-phosphate by phosphoriboisomerase. They incubated the colorimetric reagents and sample at 37°C for 30 min and read the absorbance of 540 nm, and they also introduced the use of D-ribulose *ortho*-nitrophenylhydrazone as a standard in the reaction. This derivative is crystalline and stable and reacts in the same way as free ribulose. Dickens and Williamson (1956) reported that ribulose and xylulose gave maximal and equal absorbances at 540 nm when reacted for 2 hr at room temperature, whereas ribulose and xylulose 5-phosphates required 2 hr at 37°C for the same result, and curves were published showing the time course of color development. Stumpf and Horecker (1956) reported that ribulose gave its maximal color after 15 min at room temperature, and xylulose reached its maximum after 100 min, whereas Dische and Shigeura (1957) reported that ribulose required 25 min and xylulose 180 min. After 180 min the ribulose color had decreased to 93% of its maximal value. Ribulose 5-phosphate and xylulose 5-phosphate gave equal maximal colors after 18 hr at room temperature.

Ashwell and Hickman (1957) modified the original procedure slightly so that the final volume of reactants was 6.2 ml instead of 7.4 ml, and they published standard color yields for the free ketopentoses and their phosphates (Table 3.1). A standard color yield for ribulose 5-phosphate has also been published by Wood (1975b). Trioses and triose phosphates are noteworthy in giving a blue color in the reaction (Dische and Borenfreund, 1951).

The Phloroglucinol Reaction

The phloroglucinol reaction, described by Dische and Borenfreund (1957), is relatively specific for aldopentoses and their phosphates, which give a cherry-red color with a maximum at 552 nm. Ribose 5-phosphate was reported to produce twice as much color as free ribose, while an equimolecular amount of ketopentose phosphate gave a negligible color. Hexoses and heptoses produce brown and green colors when present at much higher concentrations, and a partial correction for their presence may be obtained by recording $(A_{552} - A_{510})$. For ketopentose phosphates this corrected absorbance has been reported to be only 3% of the value of that due to an equivalent amount of ribose 5-phosphate (Dische and Shigeura, 1957).

The Orcinol Reaction

Although the orcinol reaction is relatively nonspecific, various modifications have been widely used in work on the pentose phosphate pathway, and some of them have been described by Ashwell (1957). The absorption spectra of the colored products given by ribulose, xylulose, and xylulose 5-phosphate have been published by Stumpf and Horecker (1956), and a

TABLE 3.1

Color Yields in the Cysteine-Carbazole Reaction[a]

Sample (0.1 μmol)	A_{540} in a 1-cm cell	Conditions	Final volume (ml)
Ribulose	0.495	15 min, RT[b]	6.2
Xylulose	0.250	15 min, RT	6.2
Xylulose	0.495	180 min, RT	6.2
Ribulose 5-phosphate	0.140	30 min, 37°	6.2
Xylulose 5-phosphate	0.050	30 min, 37°	6.2
Ribulose 5-phosphate[c]	0.320	120 min, 37°	7.4

[a] From Ashwell and Hickman (1957).
[b] RT indicates room temperature.
[c] Wood (1975b).

procedure applicable to a mixture of a pentose and a heptulose was described by Horecker (1957a). For pentose phosphates a modification of the method has been developed that reduces the interference from free pentoses and from hexoses and heptoses and their phosphates (Blackmore and Williams, 1974). Three different procedures using orcinol and ferric chloride have been described by Dische (1953) for the measurement of heptoses. A reaction of sedoheptulose and sedoheptulosan with orcinol alone has been described (Sonka and Kucharova, 1956; Snell *et al.*, 1961), and sedoheptulose 7-phosphate reacts similarly (Wood and Poon, 1970).

The Cysteine–Sulfuric Acid Reaction

The cysteine–sulfuric acid reaction is very sensitive toward heptoses (Dische, 1953). Sedoheptulose 1-phosphate has been reported to produce less color than the 7-phosphate (Bucolo and Bartlett, 1960).

Paper Chromatography

Methods for separating and identifying sugar phosphates have been reviewed by Benson (1955, 1957) and by Hollmann and Touster (1964), and procedures specifically applicable to intermediates of the pentose phosphate pathway and related sugars have been described by Wood (1968). The general techniques for chromatographing sugar phosphates involve the addition of EDTA to the solvents and the use of acid-washed papers to reduce the interference of metal ions. In view of the alkali lability of many of the intermediates, alkaline solvents are to be avoided, and the presence of strong acids is favored to reduce the ionization of the phosphate group, which otherwise leads to the appearance of multiple spots. However, an acetic acid–ethanol mixture was successfully used by Dische and Shigeura (1957) to separate seven-carbon and five-carbon sugar phosphates, and a similar mixture was reported to have separated ribose 5-phosphate from arabinose 5-phosphate (Horecker *et al.*, 1951). The free sugars can be separated in 80% phenol (Dickens and Williamson, 1956; Horecker *et al.*, 1956b; Wood, 1968) and in 2-propanol-*n*-butanol water (Wood, 1968). The phosphorylated intermediates have been separated in a solvent (GW3) containing a mixture of trichloracetic acid and formic acid (Wood, 1968; Williams *et al.*, 1978a) and in a solvent containing formic acid alone (Wood, 1968).

In view of the close similarities between the various phosphorylated sugars, attempts have been made to use the differences in configuration of their hydroxyl groups as a basis for separation. Cohen and Scott (Cohen and Scott, 1950; Scott and Cohen, 1951) added boric acid to an ethanol solvent and showed that the migration of ribose 5-phosphate was inhibited while

that of arabinose 5-phosphate was unaffected. They attributed the effect of boric acid to complex formation between boric acid and the *cis*-hydroxyls of ribose 5-phosphate. Other workers have obtained similar effects with the free pentoses (Mitsuhashi and Lampen, 1953), and it was reported that the presence of boric acid caused the R_f values of ribose and ribulose in acetone water to increase, while those of xylose, xylulose, and arabinose did not change (Seegmiller and Horecker, 1952). Wood and Gascon (1980) were unable to reproduce the separation of ribose 5-phosphate and arabinose 5-phosphate reported by Cohen and Scott (1950) and Williams *et al.* (1978a), but they succeeded in separating the two in other solvents containing either borate or tungstate as the complexing agent.

Detection Reagents for Paper Chromatography

Certain color reagents have proved to be particularly useful for the detection of intermediates of the pathway and their parent sugars on paper. Among these should be mentioned the phloroglucinol reagent for aldopentoses and their phosphates (Borenfreund and Dische, 1957b); an orcinol reagent for heptoses and their phosphates (Klevstrand and Nordal, 1950); 3,4-dinitrobenzoic acid for ketopentoses, ketopentose phosphates, erythrose 4-phosphate, and triose phosphates (Wood and Abrahams, 1968); and a urea–hydrochloric acid reagent for erythrose 4-phosphate (Wood, 1968). A cysteine–carbazole spray has also been described (Benvenue and Williams, 1951; Ujejski and Waygood, 1955).

Column Chromatography

Column chromatography has been extensively used for the separation, isolation, and purification of the intermediates of the pathway and of the parent sugars. The strong anion exchanger Dowex-1 has been most often employed, in the formate form, and elution has been carried out with mixtures of formic acid and either sodium or ammonium formate, with the occasional addition of borate to form complexes. Dowex-1 sulfate was used in conjunction with sodium sulfate–borate mixtures to separate four isomeric ribose phosphates (Khym *et al.*, 1954). Dowex-1 chloride has occasionally been used (Byrne and Lardy, 1954), and Dowex-1 borate has proved particularly efficacious in the separation of the free sugars obtained following phosphatase hydrolysis of the phosphorylated intermediates (Khym and Zill, 1952). Bartlett and Bucolo (1968) have published a complete system for separating as many as 20 phosphorylated compounds on Dowex-1 formate, and their system was employed by Williams *et al.* (1978a) to separate the products of ribose 5-phosphate metabolism by rat liver extracts.

In none of the systems using Dowex-1 was any separation of xylulose 5-phosphate and ribulose 5-phosphate obtained, although Dickens and Williamson (1956) did obtain a double hump in the ketopentose phosphate peak, suggesting a partial separation in their system. However, by the use of a dihydroxyboryl-cellulose column, a complete separation of these two isomers has been obtained (Gascon *et al.*, 1981). Some typical separations are listed in Table 3.2.

Other Separation Methods

Very little use has been made of thin-layer chromatography in this field, but high-voltage electrophoresis on paper was used by Vanderheiden (1964, 1965) to separate and identify heptulose and octulose bisphosphates in human erythrocytes. Separations by HPLC have been described of dansylated intermediates of the pathway (Hino and Minakami, 1983) and of the ribose phosphates (Chandrasekaran and Ardalan, 1983).

PREPARATION AND GENERAL CHEMISTRY

Synthesis Using Aldolase Condensation

The procedure of synthesis using aldolase condensation has been successfully used to produce phosphorylated ketoses with the phosphate group in the 1-position or to synthesize bisphosphates with one of the phosphates on C-1. As pointed out by Byrne and Lardy (1954) the newly formed hydroxyl groups are trans to each other and are in the same relative configuration as those at the 3- and 4-positions of fructose. Thus, glycolaldehyde was condensed with dihydroxyacetone phosphate to form D-xylulose 5-phosphate (Byrne and Lardy, 1954). When glycolaldehyde phosphate was used instead of glycolaldehyde, the D-xylulose 1,5-bisphosphate was produced (Byrne and Lardy, 1954; Racker and Schroeder, 1958; McCurry and Tolbert, 1977). D-Erythrose when condensed with dihydroxyacetone phosphate gave D-*altro*-heptulose 1-phosphate (Horecker *et al.*, 1953). Paoletti *et al.* (1979a) have used the technique to prepare D-*glycero*-D-*altro*-octulose 1,8-bisphosphate and D-*glycero*-D-*ido*-octulose 1,8-bisphosphate. These syntheses are illustrated in Fig. 3.1.

Oxidation with Bromine and Iodine

Aldopentose phosphates are readily oxidized by alkaline aqueous bromine or iodine solutions, while ketopentose phosphates are relatively resist-

TABLE 3.2

Column Chromatography of Sugar Phosphates and Free Sugars

Eluting solvent	Order of elution	Reference
Dowex-1 formate		
0.1 *M* formic/30 m*M* formate, pH 3.1	R-5-P, Ru-5-P	Horecker *et al.* (1951), Srere *et al.* (1958), Seegmiller and Horecker (1952)
0.2 *M* formic/30 m*M* formate	S-7-P, R-5-P, Ru-5-P	Horecker *et al.* (1953)
0.2 *M* formic/20 m*M* formate	R-5-P + F-6-P, Ara-5-P	Volk (1966)
Gradient to 0.2 *M* formic/500 m*M* formate	S-7-P, S-1,7-PP, F-1,6-PP	Smyrniotis and Horecker (1956)
Gradient to 0.4 *M* formic/100 m*M* formate	Ru-5-P + Xu-5-P, Ru-1,5-PP	Stumpf and Horecker (1956)
500 m*M* formate/0.5 m*M* borate	Ru-5-P + Xu-5-P, R-5-P	Dickens and Williamson (1956)
0.1 *M* formic/250 m*M* formate	Octu-8-P, F-6-P, R-5-P	Racker and Schroeder (1957)
Gradient to 1 *M* NH_4 formate, pH 3	5-7-P, S-1,7-PP, F-1,6-PP	Bucolo and Bartlett (1960)
0.2 *M* formic/2 *M* formate	Er-4-P	Simpson *et al.* (1966)
Dowex-1 sulfate		
sodium sulfate/borate	R-4-P, R-2-P, R-3-P, R-5-P	Khym *et al.* (1954), Benson (1957)
Dowex-1 chloride		
0.025 *N* hydrochloric acid	P_i, triose-P	Byrne and Lardy (1954)
0.100 *N* hydrochloric acid	R-5-P, F-1,6-PP	Byrne and Lardy (1954)
Dowex-1 borate		
15 m*M* borate	Rib, Ara, Xyl	Khym and Zill (1952), Tabachnik *et al.* (1958)
20–40 m*M* borate	Rib + Xu, Ru, Ara, Xyl	Lampen (1953), Ashwell and Hickman (1954), Dickens and Williamson (1956)
Dihydroxyboryl-cellulose		
2 *M* LiCl + additions	R-5-P, Ru-5-P, Xu-5-P	Gascon *et al.* (1981)
0.2 *M* $MgCl_2$	Ara-5-P, R-5-P	Gascon *et al.* (1981)
DEAE-Sephadex A-25		
Gradient to 0.2 *M* LiCl	S-7-P, Ru-5-P + Xu-5-P, R-5-P	Wood and Poon (1970), T. Wood (unpublished data)
Gradient of acetic acid, pH 4.5	—	Serianni *et al.* (1979)
DEAE-Cellulose		
Gradient to 0.2 *M* LiCl	6-PG, Ru-1,5-PP	Kuehn and Hsu (1978)

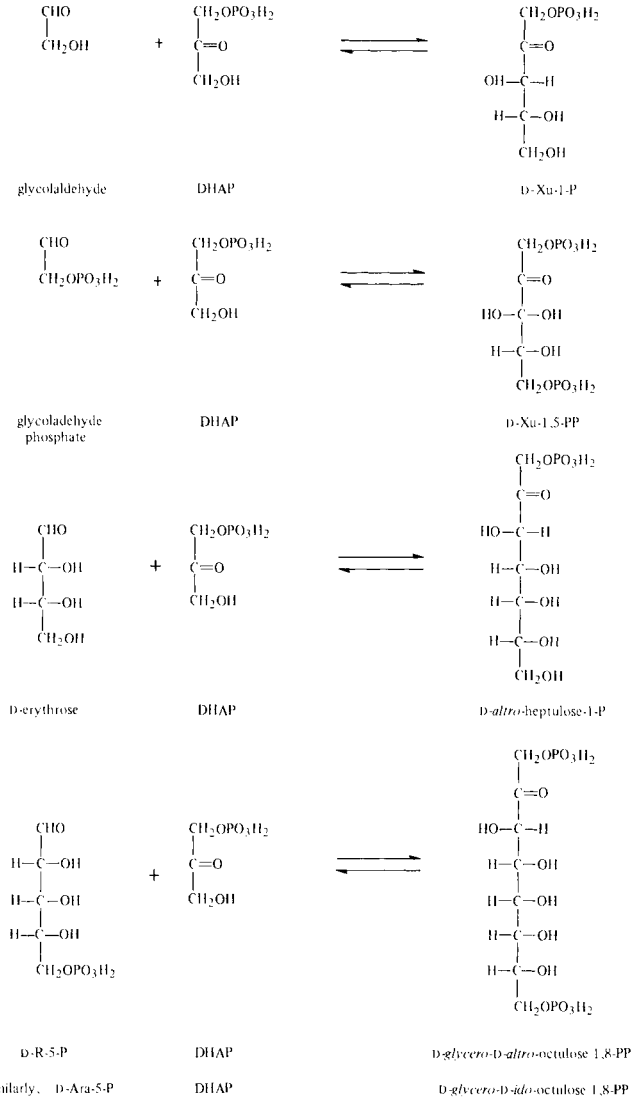


Fig. 3.1 Synthesis of mono- and bisphosphates by aldolase condensation.

ant (Lampen, 1953; Dickens and Williamson, 1956; Ashwell, 1957). These properties have been used to remove aldopentose phosphates from mixtures of the aldopentose and ketopentose phosphates (Lampen, 1953) and to prepare 6-phosphogluconate and 5-phosphoribonate (Glock, 1952). Time courses for the reaction of ribose 5-phosphate and ribulose 5-phos-

phate with bromine have been published by Seegmiller and Horecker (1952), and the procedure for the oxidation of glucose 6-phosphate to 6-phosphogluconate has been described in detail by Horecker (1957b). The oxidation of ribose 5- phosphate with iodine was described by De la Haba *et al.* (1955).

Stability of the Pentose Phosphates and Parent Sugars

Even in the solid state, ribose 5-phosphate, at temperatures near freezing, very slowly produces small amounts of ribulose 5-phosphate (Cohen, 1953; Axelrod and Jang, 1954; Hurwitz and Horecker, 1956). Similarly, xylose 5-phosphate is slowly transformed to xylulose 5-phosphate (Moffat and Khorana, 1956; Serianni *et al.*, 1982), and frozen solutions of D-ribulose formed D-arabinose (Cohen, 1953). The conversion of ribose 5-phosphate to ribulose 5-phosphate was noted by Dickens and Williamson (1956), who introduced a procedure of alkaline treatment to destroy the contaminating ketopentose phosphate. The spontaneous conversion of ribulose 1,5-bisphosphate to the xylulose 1,5-bisphosphate has been described (Wong *et al.*, 1982), and 3-phosphoglyceric acid, phosphoerythronic acid, and phosphoglycolic acid have been reported among the breakdown products of the former when acidic paper chromatograms were stored (Kauss and Kandler, 1964).

The alkaline labilities of ribulose and ribulose 5-phosphate at 37°C, pH 8.3, and pH 9.8 were studied by Cohen (1953), who reported that the major product in each case no longer reacted as a ketopentose in the cysteine–carbazole reaction but gave a reaction with orcinol similar to that of arabinose. He suggested that ribulose was more stable than its phosphate because the free sugar can exist in the furanose ring form, while the phosphate is in the open-chain configuration. Decomposition of both was retarded by complex formation with borate at pH 8. Simpson and Wood (1958) have published data showing the rate of destruction of ribose 5-phosphate, ribulose 5-phosphate, fructose 6-phosphate, and fructose 1,6-bisphosphate by 0.5 *N* alkali at room temperature and by 1.0 *N* sulfuric acid at 90°C, and Hurwitz and Horecker (1956) investigated the breakdown of ribose 5-phosphate, ribulose 5-phosphate, and xylulose 5-phosphate by 1 *N* sodium hydroxide at 25°C. The factors influencing the alkali lability of ribose and the various ribose phosphates have been discussed by Khym *et al.* (1954), who published data showing the breakdown of ribose, ribose 2-phosphate, and ribose 5-phosphate in 0.01 *N* sodium hydroxide at room temperature. Axelrod and Jang (1954) have shown the destruction of ribulose 5-phosphate by 0.1 *M* sodium carbonate over 60 min and the simultaneous formation of a compound with an absorption maximum at 310 nm.

The stability of ribulose 5-phosphate, xylulose 5-phosphate, and xylulose in various buffers over the range pH 7.0–9.5 has been studied (Borenfreund and Dische, 1957a). After exposure to morpholine–chloride buffer at pH 8.7 for 3 hr at room temperature, 90% of ribulose 5-phosphate was recovered, as measured by enzymatic assay, and at a pH of 9.2, no noticeable loss of xylulose 5-phosphate was observed (Gascon *et al.*, 1981).

Dickens and Williamson (1956) measured the rate of hydrolysis of ribulose 5-phosphate and xylulose 5-phosphate by 1 *N* sulfuric acid at 100°C and obtained a hydrolysis constant in agreement with the half-life of 50 min for ribulose 5-phosphate previously reported (Horecker *et al.*, 1951). Xylulose 5-phosphate, ribulose 5-phosphate, ribose 5-phosphate, and 6-phosphogluconate were found to be stable in 0.6 *N* perchloric acid for 2 hr at 60°C in simulated tissue extracts. However, in neutralized extracts at pH 6.8, approximately 50% of the ketopentose phosphates had disappeared after 2 hr at 60°C, although no loss of ribose 5-phosphate or 6-phosphogluconate was detected (Kauffman *et al.*, 1969).

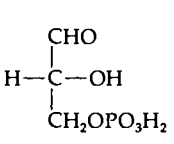
Structure of the Sugar Phosphates in Solution

Gray and Barker (1970) have shown that a number of ketose phosphates that cannot exist in ring forms are present in solution as the open-chain keto form, and Serianni *et al.* (1979) have described the structure of a number of aldose phosphates in solution and listed the percentage of hydrate and α and β forms for all four of the aldopentose 5-phosphates.

The structures of all the major contributing forms of D-*altro*-heptulose 1,7-bisphosphate and D-*glycero*-D-*altro*- and D-*glycero*-D-*ido*-octulose 1,8-bisphosphates have been established by ^{13}C-NMR spectroscopy (Franke *et al.*, 1984).

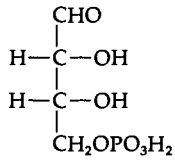
INDIVIDUAL INTERMEDIATES

D-Glyceraldehyde 3-Phosphate



The preparation and properties of D-glyceraldehyde 3-phosphate have been described by Lardy (1957) and the properties and assay by Beck (1957). In solution it exists mainly as the monomeric hydrate (Serianni *et al.*, 1979).

D-Erythrose 4-Phosphate



Preparation

The chemical synthesis by direct phosphorylation of a protected derivative of D-erythrose to give D-erythrose 4-phosphate dimethylacetal was described by Ballou (1963). The procedure is long and complex, and the simpler direct oxidation of glucose 6-phosphate with lead tetraacetate has been preferred and widely used. A chemical method involving hydrogenolysis of aldonitrile phosphates prepared from the next lowest homolog has been described and applied to the synthesis of ^{13}C-enriched sugar phosphates, including the D-erythrose derivative (Serianni *et al.*, 1979, 1982).

The direct oxidation of D-glucose 6-phosphate with lead tetraacetate produced D-erythrose 4-phosphate, which was separated on a Dowex-1 formate column and isolated as the barium salt of the stable hydrazone (Ballou, 1963; Simpson *et al.*, 1966; Baxter *et al.*, 1959; Sieben *et al.*, 1966). This procedure has also been employed to prepare analogs of D-erythrose 4-phosphate, namely D-erythrose 4-phosphonate by the oxidation of D-glucose 6-phosphonate and D-erythrose 4-homophosphonate from D-glucose 6-homophosphonate (Le Marechal *et al.*, 1980).

Properties

D-Erythrose 4-phosphate is similar to D-glyceraldehyde 3-phosphate in its sensitivity to acid, being 50% hydrolyzed in 20 min by 1 *N* acid at 100°C. On treatment with 1 *N* sodium hydroxide at room temperature, half, but not all, the available phosphate is split off as inorganic phosphate (Ballou, 1963). The compound has been reported to be stable over a 4-hr period at the pH of distilled water (Blackmore *et al.*, 1976) but unstable at pH values above 6 (Williams *et al.*, 1980; Duke *et al.*, 1981). Serianni *et al.* (1979) found it to be stable on long-term storage at −15°C at pH 1–2, and Duke *et al.* (1981) stated that frozen solutions at pH 1 could be stored indefinitely at −20°C. The usual methods of tissue extraction with ethanol or acid did not appear to decompose it (Williams *et al.*, 1980), but neutralization of a perchloric acid extract and the presence of tris buffer at pH 7.4 caused some decomposition (Blackmore *et al.*, 1976).

The specific rotation varies with wavelength but is very small, and values have been reported by Ballou (1963). Erythrose 4-phosphate has been re-

ported to form dimers in aqueous solution, and as a result, double spots appear after chromatography on paper and double peaks when columns are used (Blackmore *et al.*, 1976; Williams *et al.*, 1980). At concentrations around 1.0 *M* the compound is present as an equilibrium mixture of the monomeric aldehyde, the monomeric hydrated aldehyde, and some 60% of three dimeric forms. Below 0.04 *M* the hydrated monomer was predominant and no dimers could be detected (Serianni *et al.*, 1979; Duke *et al.*, 1981).

The biochemistry of D-erythrose 4-phosphate has been reviewed by Williams *et al.* (1980).

Analysis

D-Erythrose 4-phosphate may be estimated colorimetrically using a phenol-sulfuric acid reagent (Sieben *et al.*, 1966) or the cysteine–sulfuric acid–fructose method (Dische and Dische, 1958). The most commonly employed method of enzymatic analysis is that described by Cooper *et al.* (1958). The principle is illustrated by Eqs. (1) and (2).

$$\text{D-Er-4-P} + \text{D-F-6-P} \xrightleftharpoons{\text{TA}} \text{D-S-7-P} + \text{D-GAP} \quad (1)$$

$$\text{D-GAP} + \text{NADH} + \text{H}^+ \xrightleftharpoons{\text{TPI/GlPDH}} \text{GlP} + \text{NAD}^+ \quad (2)$$

The glyceraldehyde 3-phosphate that is formed oxidizes NADH to NAD, and a change in absorbance at 340 nm is produced. It was pointed out by Paoletti *et al.* (1979c) that the previous method was not specific for D-erythrose 4-phosphate, as other aldoses and aldose phosphates could serve as acceptors of the C_3 group and might be mistaken for erythrose 4-phosphate. They introduced two new assays, which they claimed to be much more specific.

Assay I

$$\text{D-Er-4-P} + \text{D-Fru} \xrightleftharpoons{\text{TK}} \text{D-F-6-P} + \text{erythrose} \quad (3)$$

$$\text{D-F-6-P} + \text{NADP}^+ \xrightleftharpoons{\text{PGI/G-6-PDH}} \text{6-PG} + \text{NADPH} + \text{H}^+ \quad (4)$$

Assay II

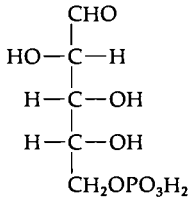
$$\text{D-Er-4-P} + \text{3-hydroxypyruvate} \xrightleftharpoons{\text{TK}} \text{D-F-6-P} + \text{CO}_2 \quad (5)$$

$$\text{D-F-6-P} + \text{NADP}^+ \xrightleftharpoons{\text{PGI/G-6-PDH}} \text{6-PG} + \text{NADPH} + \text{H}^+ \quad (6)$$

D-Erythrose 4-phosphate reacts slowly with D-glyceraldehyde 3-phosphate dehydrogenase and can be assayed in the presence of NAD, arsenate,

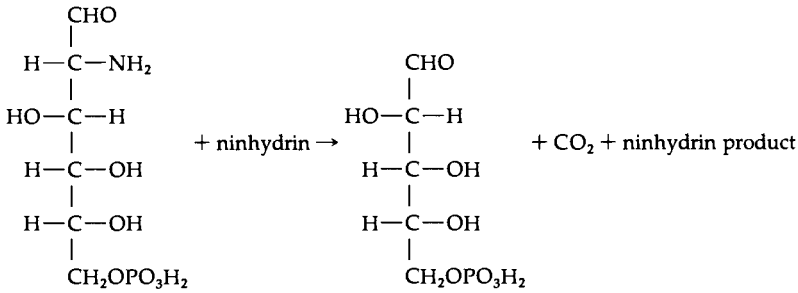
and relatively large amounts of the enzyme in a similar manner to D-glyceraldehyde phosphate itself (Racker *et al.*, 1959).

D-Arabinose 5-Phosphate



Preparation

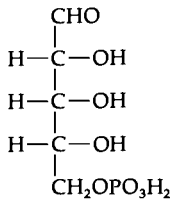
D-Arabinose 5-phosphate may be prepared from D-arabinose and polyphosphoric acid (Seegmiller and Horecker, 1952; Levin and Racker, 1959) or by treating D-glucosamine 6-phosphate with ninhydrin (Volk, 1966). The reaction is as follows:



Properties and Analysis

D-Arabinose 5-phosphate has been suggested to be an intermediate in a new enlarged pentose phosphate pathway (Williams *et al.*, 1978a). Its properties would be expected to be similar to those of ribose 5-phosphate. In solution 58% is present as the α-furanose form, 40% as the β-furanose, and 2% as the straight-chain hydrate (Serianni *et al.*, 1979). It is relatively stable to acid, only 16% of the phosphate being split off by 1 *N* sulfuric acid at 100°C in 1 hr (Volk, 1960). No specific assay method or color reaction appears to be known, but reactions with orcinol and phloroglucinol have been recorded (Williams *et al.*, 1978a; Wood and Gascon, 1980). It may be separated from ribose 5-phosphate by chromatography on paper and on columns (Volk, 1966; Williams *et al.*, 1978a; Wood and Gascon, 1980; Gascon *et al.*, 1981).

D-Ribose 5-Phosphate



Preparation

Two methods of preparation of D-ribose 5-phosphate have been described by Horecker (1957c) and by Gross *et al.* (1983). For example,

$$\text{D-ribose} + \text{ATP} \xrightarrow{\text{ribokinase}} \text{D-R-5-P} + \text{ADP}$$

$$\text{AMP} \xrightarrow{1\ N\ \text{HCl, 30 min, 100°C}} \text{D-R-5-P} + \text{adenine}$$

The synthesis of ^{13}C-enriched ribose 5-phosphate was described by Serianni *et al.* (1979).

Properties

The stability to acid and alkaline conditions, the oxidation by bromine, and the slow transformation to ribulose 5-phosphate have already been described. A number of values for the specific rotation have been reported, +16.5° (Levene and Stiller, 1934; Michelson and Todd, 1949), +17° (Schlenk, 1942), and +22.8° (Horecker *et al.*, 1951).

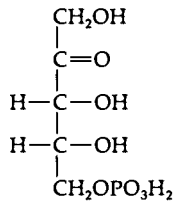
Ribose 5-phosphate undergoes a direct reaction with cysteine and aminomethylmercaptan in solution, and this may cause complications in assay mixtures containing the phosphorylated sugar and thiol compounds; however, no reaction with glutathione was observed (Dische, 1958). The periodate oxidation of ribose 5-phosphate was investigated and shown to proceed through the intermediate formation of a formic ester (Bernofsky and Wanda, 1982). In solution it exists 34% as the α-furanose form, 64% as the β-furanose, and approximately 1% as the straight-chain hydrate; consequently, it shows no carbonyl absorption in the 280-nm region (Serianni *et al.*, 1979; Knowles *et al.*, 1980).

Analysis

Ribose 5-phosphate can be estimated by the orcinol reaction and measured in the presence of ketopentose phosphates with phloroglucinol. It may be assayed enzymatically after destruction of ketopentose phosphates with alkali (Cooper *et al.*, 1958), or total pentose phosphate may be measured with transketolase, D-ribose-5-phosphate ketol-isomerase (isomer-

ase), and D-ribulose-5-phosphate 3-epimerase (epimerase), and then the total ketopentose phosphate (measured with transketolase and epimerase) subtracted to give the ribose 5-phosphate (Wood, 1975b).

D-Ribulose 5-Phosphate



Preparation

Methods for the preparation of D- and L-ribulose 5-phosphates have been reviewed by Simpson and Wood (1958) and by Simpson (1966). D-Ribulose may be phosphorylated with ATP and a specific kinase [Eq. (7)], or D-ribulose 5-phosphate may be prepared directly by dehydrogenation and decarboxylation of 6-phosphogluconate [Eqs. (8) and (9)].

$$\text{D-Ru} + \text{ATP} \xrightarrow{\text{D-ribulokinase}} \text{D-Ru-5-P} + \text{ADP} \tag{7}$$

$$\text{6-PG} + \text{NADP}^+ \xrightarrow{\text{6-PGDH}} \text{D-Ru-5-P} + \text{CO}_2 + \text{NADPH} + \text{H}^+ \tag{8}$$

$$\text{pyruvate} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NADP}^+ \tag{9}$$

Since neither D-ribulose nor the specific kinase are readily available, the second method has been most widely used. An early procedure described by Horecker's group (Horecker and Smyrniotis, 1950; Horecker *et al.*, 1951; Horecker, 1957d) gave a mixture of ribulose 5-phosphate and ribose 5-phosphate due to the presence of isomerase in the enzymes used. In a later procedure (Pontremoli *et al.*, 1961a; Pontremoli and Mangiarotti, 1962; Pontremoli, 1966) the lithium salt of D-ribulose 5-phosphate was prepared and appeared to be free from ribose 5-phosphate and xylulose 5-phosphate. In a modification of the previous procedures, the stable barium salt of the hydrazone of D-ribulose 5-phosphate was prepared. Only lactate dehydrogenase from beef heart was found to be sufficiently free from D-ribulose-5-phosphate 3-epimerase to be suitable for the regeneration of NADP by the reaction of Eq. (9) (Wood, 1975d).

D-Ribulose 5-phosphate may also be very simply prepared by the action of D-ribose-5-phosphate ketol-isomerase on its substrate to form an equilibrium mixture containing D-ribose and D-ribulose 5-phosphates. Such a mixture may be used directly for the assay of D-ribulose-5-phosphate 3-epi-

merase (Wood, 1975b) or further purified. Thus, Srere *et al.* (1958) oxidized the remaining ribose 5-phosphate with bromine and isolated the ribulose 5-phosphate by ion-exchange chromatography. An improved procedure for the separation of D-ribulose 5-phosphate from such an equilibrium mixture by column chromatography has been published by Gascon *et al.* (1981).

The preparation of D-[1-^{3}H]-ribulose 5-phosphate from 6-phosphogluconate has been described by Lienhard and Rose (1964).

Properties

The behavior of D-ribulose 5-phosphate in acid and alkaline media and its resistance to bromine oxidation have already been described. A preparation contaminated with 15–20% D-phosphate had $[\alpha]_D$ of $-28°$ to $-32°$ (Horecker *et al.*, 1953; Hurwitz *et al.*, 1956), and Horecker (1957d) has reported $-40°$ for the pure compound. This may be compared with a value of $+29°$ for L-ribulose 5-phosphate (Simpson and Wood, 1958).

A number of ketose phosphates that cannot exist in ring forms in solution are present in the open-chain keto form and absorb in the ultraviolet region (Gray and Barker, 1970). Ribulose 5-phosphate has such an absorbance with a peak at 280 nm and a molar extinction coefficient variously reported as 58.6 (Knowles, 1968; Knowles *et al.*, 1969) and 85 (Wood, 1975d). The initial appearance of the chromophore is followed by further absorbance changes (Knowles, 1968; Knowles and Pon, 1968a; Knowles *et al.*, 1980) that have been attributed to the formation of dimers and rearrangements of the molecule (Wood, 1975d). The circular dichroism (CD) and optical rotatory dispersion (ORD) spectra of ribulose 5-phosphate and ribose 5-phosphate have also been measured (Wood, 1975d; Karmeli *et al.*, 1983). The increase in the absorbance in the range 280–290 nm has been employed as the basis of an assay for ribose-5-phosphate ketol-isomerase (Knowles *et al.*, 1969; Wood, 1970, 1975c). Another compound, 4-hydroxy-5-methyl-3(2H)-furanone is also formed by isomerase acting on ribose 5-phosphate and may have arisen from ribulose 5-phosphate by dehydration and elimination of the phosphate group (Knowles *et al.*, 1980; see Fig. 3.2). By contrast, the spectra of ribulose 1,5-bisphosphate and of xylulose 5-phosphate do not change with time (Wood, 1975d).

Analysis

In the absence of other ketopentose phosphates the compound may be measured by the cysteine–carbazole reaction with ribulose *ortho*-nitrophenylhydrazone as a standard. Alternatively, the concentration may be calculated from the fact that 0.1 μmol gives an absorbance of 0.32 at 540 nm in a 1-cm cell, in a volume of 7.4 ml, after a heating time of 2 hr at 37°C

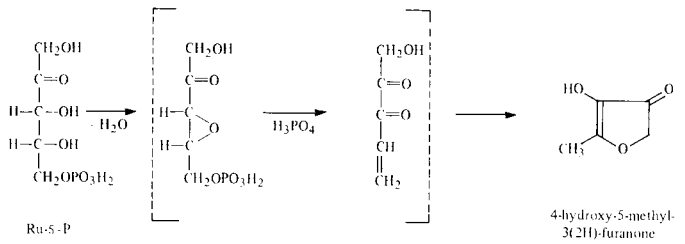


Fig. 3.2 Formation of 4-hydroxy-5-methyl-3(2H)-furanone from ribulose 5-phosphate (Knowles *et al.*, 1980).

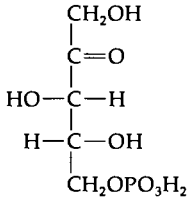
(Wood, 1975b). In mixtures with other compounds enzymatic methods must be used. Thus, D-xylulose 5-phosphate is first estimated using transketolase [Eqs. (10) and (11)], and then ribulose 5-phosphate is measured by adding D-ribulose-5-phosphate 3-epimerase (Cooper *et al.*, 1958; Wood, 1975b).

$$\text{D-Xu-5-P} + \text{D-R-5-P} \overset{\text{TK}}{\rightleftharpoons} \text{D-S-7-P} + \text{D-GAP} \tag{10}$$

$$\text{D-GAP} + \text{NADH} + \text{H}^+ \overset{\text{TPI/GlPDH}}{\rightleftharpoons} \text{GlP} + \text{NAD}^+ \tag{11}$$

$$\text{D-Ru-5-P} \overset{\text{epimerase}}{\rightleftharpoons} \text{D-Xu-5-P} \tag{12}$$

D-Xylulose 5-Phosphate



Preparation

D-Xylulose 5-phosphate has been prepared by direct phosphorylation of D-xylulose using a D-xylulokinase prepared either from *Aerobacter aerogenes* (Simpson and Bhuyan, 1962; Simpson, 1966) or *Lactobacillus pentosus* (Stumpf and Horecker, 1956). In the latter case the kinase was contaminated with epimerase, so the ribulose 5-phosphate was removed by phosphorylation with ATP and phosphoribulokinase and the resulting D-ribulose 1,5-bisphosphate separated from D-xylulose 5-phosphate on a Dowex-1 formate column. In another procedure a mixture of ribose 5-phosphate, ribulose 5-phosphate, and xylulose 5-phosphate was prepared from ribose

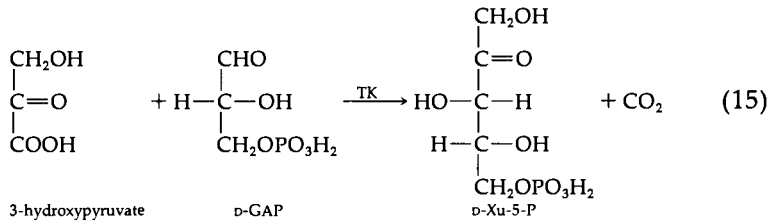
5-phosphate by the action of the *Lactobacillus pentosus* epimerase and spinach isomerase [Eq. (13)]. The mixture was treated with ATP and phosphoribulokinase, which in the presence of isomerase converted ribose 5-phosphate and ribulose 5-phosphate to the 1,5-bisphosphate [Eq. (14)]. The bisphosphate and xylulose 5-phosphate were separated from each other by barium precipitation (Horecker *et al.*, 1957).

$$\text{R-5-P} \overset{\text{isomerase}}{\rightleftharpoons} \text{Ru-5-P} \overset{\text{epimerase}}{\rightleftharpoons} \text{Xu-5-P} \tag{13}$$

$$\text{Ru-5-P} + \text{ATP} \xrightarrow{\text{phosphoribulokinase}} \text{Ru-1,5-PP} + \text{ADP} \tag{14}$$

The preparation of equilibrium mixtures [Eq. (13)] using spinach isomerase and yeast epimerase has been described by Wood (1975b). Such mixtures may either be employed directly for the assay of transketolase or the D-xylulose 5-phosphate separated by column chromatography and precipitated as the barium salt or the barium salt of the hydrazone (Gascon *et al.*, 1981).

A simple and most popular method of preparing D-xylulose 5-phosphate is the reaction of 3-hydroxypyruvate with D-glyceraldehyde phosphate in the presence of transketolase [Eq. (15)].



(15)

This procedure was first employed by De la Haba *et al.* (1955) and subsequently by a number of other workers (Srere *et al.*, 1958; Wood, 1975d). Contamination of the transketolase with epimerase and isomerase will result in the xylulose 5-phosphate being converted to ribulose- and ribose-5-phosphates, and an improved procedure has been published allowing the progress of the reaction to be followed and the reaction halted before appreciable side reactions have occurred (Wood, 1973b).

Properties

The properties of D-xylulose 5-phosphate are very similar to those of D-ribulose 5-phosphate; however, it is more stable, and the ultraviolet absorption spectrum does not change with time (Wood, 1975d). The CD spectrum was the inverse of that of D-ribulose 5-phosphate (Karmali *et al.*, 1983). Some 84% of the barium salt of the hydrazone remained intact after 48 hr at 78°C *in vacuo*, and 94% was recovered after exposure to 1 *N* sodium hydroxide at 30°C for 1 hr (Simpson and Bhuyan, 1962).

Analysis

In the absence of other ketopentose phosphates, D-xylulose 5-phosphate may be estimated colorimetrically by the cysteine–carbazole reaction. In mixtures with other compounds it may most readily be determined by reaction with transketolase in the presence of excess ribose 5-phosphate as an acceptor (see the section on ribulose 5-phosphate for the equations) (Ashwell and Hickman, 1957; Cooper *et al.*, 1958; Wood, 1975b). Kochetov (1982d) has described an assay using ferricyanide and transketolase, but rather large amounts of the enzyme are required.

D-Xylulose 5-phosphate may also be assayed by the sequence of reactions described by Horecker (1962b) [Eqs. (16) and (17)].

$$\text{D-Xu-5-P} + {}^{32}\text{P}_i \xrightarrow{\text{phosphoketolase}} \text{acetyl-[}^{32}\text{P]phosphate} + \text{D-GAP} \qquad (16)$$

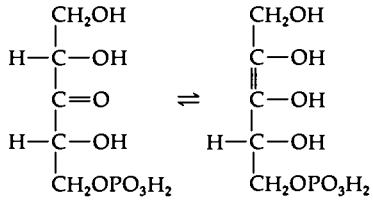
$$\text{acetyl-[}^{32}\text{P]phosphate} + \text{ADP} \xrightarrow{\text{acetokinase}} \text{acetate} + [^{32}\text{P]ATP} \qquad (17)$$

The [^{32}P]ATP is absorbed onto charcoal and the radioactivity counted. Alternatively, the glyceraldehyde phosphate may be measured by the addition of NADH and a mixture of triose-phosphate isomerase (TPI) and glycerophosphate dehydrogenase. Unfortunately, phosphoketolase is not commercially available. A somewhat cumbersome procedure was used by Hurwitz and Horecker (1956), who first hydrolyzed the phosphate to the free sugar and then rephosphorylated it with ATP plus a kinase and measured the ADP produced [Eqs. (18) and (19)].

$$\text{D-Xu-5-P} \xrightarrow{\text{phosphatase}} \text{D-Xu} + \text{P}_i \qquad (18)$$

$$\text{D-Xu} + \text{ATP} \xrightarrow{\text{xylulokinase}} \text{D-Xu-5-P} + \text{ADP} \qquad (19)$$

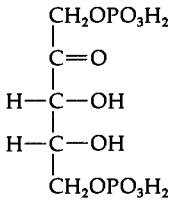
3-Ketopentose Phosphate



Following the action of a mouse spleen extract on ribose 5-phosphate, the products were hydrolyzed with phosphatase and the free sugars separated on a Dowex-1 borate column. A substance in one of the peaks obtained gave an orcinol reaction and a blue color in the cysteine–carbazole reaction. After reduction with borohydride, both ribitol and xylitol were obtained, and periodate oxidation indicated that it was a 3-ketopentose. It was sug-

gested that the product was D-*erythro*-3-pentulose, possibly arising from a 2,3-enediol phosphate intermediate in the ribose-5-phosphate isomerase reaction (Ashwell and Hickman, 1955). A similar compound was isolated by Dickens and Williamson (1956) from the incubation mixture of ribose 5-phosphate and partially purified rabbit muscle enzymes. Dische and Shigeura (1957) obtained a compound from incubation mixtures of ribose 5-phosphate with blood hemolysates that reacted with iodine at pH 5 and was converted to pentose phosphates by the hemolysate. They tentatively identified it as a 2,3-enediol-pentose 5-phosphate. The formation of similar intermediates has been suggested by Knowles and Pon (1968) and Knowles *et al.* (1980) to accompany the action of spinach isomerase on ribose 5-phosphate.

D-Ribulose 1,5-Bisphosphate



Preparation

The synthesis of D-ribulose 1,5-bisphosphate from D-ribulose 5-phosphate using phosphoribulokinase and ATP was described by Horecker *et al.* (1956a). A similar procedure gave D-xylulose 5-phosphate in addition (Horecker *et al.*, 1957). The production of the ^{14}C-labeled compound from labeled glucose has been described (Wishnick and Lane, 1969; Kuehn and Hsu, 1978), and highly detailed procedures for its production from glucose or from adenosine monophosphate have been presented (Wong *et al.*, 1982).

Properties

Ribulose bisphosphate is an unstable compound (half-life of 48 hr at pH 8 and 30°C) and isomerizes rapidly and spontaneously to xylulose bisphosphate in solution and in the form of the solid barium salt. It is stable for several months at −20°C. It should not be exposed to a pH above 8.0 (Wong *et al.*, 1982). Both phosphate groups are equally labile in acid and in alkali. Thus, at 25°C the half-life is 2.8 min in 1 *N* sodium hydroxide and 15 min in 0.1 *N* sodium hydroxide. At 100°C the half-life is 20 min in 1 *N* sulfuric acid compared to a value of 40 min for ribulose 5-phosphate. It appears that the presence of the phosphate group on C-1 renders the 5-po-

sition more labile. It is slowly cleaved by aldolase at a rate one-fiftieth of the rate with fructose 1,6-bisphosphate. It is not acted on by spinach transketolase, but one equivalent of inorganic phosphate is slowly split off by a spinach phosphatase (Horecker *et al.*, 1956a). It has an absorbance peak at 282 nm, with a molar absorption coefficient of 85.4. In solution 84% is present as the keto form (Gray and Barker, 1970), and its spectrum does not change with time (Wood, 1975d). When left on acidic paper chromatograms for 5 weeks, it was slowly destroyed, and the products were tentatively identified as 3-phosphoglyceric acid (3-PGA), phosphoerythronic acid, and phosphoglycolic acid (Kauss and Kandler, 1964).

Analysis

In solutions free from other ketopentose phosphates D-ribulose 1-5-bisphosphate may be measured using the cysteine–carbazole reaction. An enzymatic assay has been described by Racker [see Bergmeyer (1974)] as follows:

$$\text{D-Ru-1,5-PP} + CO_2 \xrightarrow[\text{carboxylase}]{\text{Ru-1,5-PP}} \text{D-3-PGA} \tag{20}$$

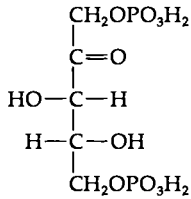
$$\text{D-3-PGA} + \text{ATP} \xrightarrow{\text{PGK}} \text{D-1,3-PPGA} \tag{21}$$

$$\text{D-1,3-PPGA} + \text{NADH} + H^+ \xrightleftharpoons{\text{GAPDH}} \text{D-GAP} + \text{NAD}^+ + P_i \tag{22}$$

$$\text{D-GAP} + \text{NADH} + H^+ \xrightarrow{\text{TPI/GlPDH}} \text{GlP} + \text{NAD}^+ \tag{23}$$

The previous assay has been described in detail together with a radiometric method involving the fixation of radioactive carbon dioxide (Latzko and Gibbs, 1972; Wong *et al.*, 1982).

D-Xylulose 1,5-Bisphosphate



Preparation and Properties

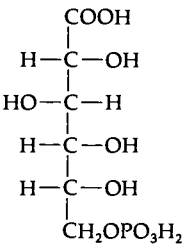
The preparation of D-xylulose 1,5-bisphosphate by condensation of glycolaldehyde phosphate and dihydroxyacetone phosphate in the presence of aldolase has already been described. It is both acid and alkali labile, being

44% hydrolyzed by 1 *N* sodium hydroxide in 15 min at room temperature; $[\alpha]_D = +10°$ (Byrne and Lardy, 1954). It is a powerful inhibitor of ribulose 1,5-bisphosphate carboxylase (K_m for ribulose bisphosphate = 20 μM; K_i for xylulose bisphosphate = 3 μM) (Wong *et al.*, 1982).

Analysis

D-Xylulose 1,5-bisphosphate may be assayed by a procedure in which it is split by aldolase and the triose phosphate formed is measured (Wong *et al.*, 1982).

D-6-Phosphogluconic Acid



Preparation

D-6-phosphogluconic acid is prepared by bromine oxidation of D-glucose 6-phosphate or by the action of glucose-6-phosphate dehydrogenase (Robison and King, 1931; Horecker, 1957b). The primary oxidation product is 6-phosphoglucono-Δ-lactone, formed by direct removal of two hydrogen atoms from the pyranose ring of D-glucose 6-phosphate. The lactone may be trapped in hydroxylamine and measured by the procedure of Hestrin (Cori and Lipmann, 1952). Above pH 6 the lactone hydrolyzes spontaneously, with a half-life reported as 1.5 min at pH 7.4 (Horecker and Smyrniotis, 1953b) and 7 min at pH 7.0 (Schofield and Sols, 1976). This hydrolysis is also catalyzed by a specific 6-phosphogluconolactonase (Schofield and Sols, 1976). The lactone is readily reduced back to glucose 6-phosphate by NADPH and glucose 6-phosphate dehydrogenase (Horecker and Smyrniotis, 1953b).

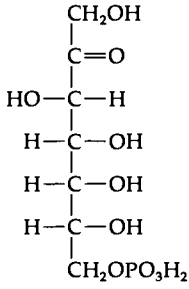
Properties

The barium salt is sparingly soluble in water (0.71 g/100 ml). The specific rotation at 5461 Å is $-1.5°$ for the barium salt and $+0.2°$ for the acid barium salt. The specific rotation of the lactone is $+21°$ (Robison and King, 1931).

Analysis

D-6-Phosphogluconic acid is usually measured enzymatically by reaction with D-6-phosphogluconate dehydrogenase and NADP (Horecker, 1957b; Bergmeyer, 1974).

D-Sedoheptulose 7-Phosphate (D-*Altro*-Heptulose 7-Phosphate)



Preparation

D-Sedoheptulase 7-phosphate has been isolated from the leaves of *Sedum spectabile* (Ujejski and Waygood, 1955). A chemical synthesis from 2-nitroethanol and D-ribose 5-phosphate has been published (McFadden *et al.*, 1965). It has been produced, mixed with sedohepulose 1,7-bisphosphate and other compounds, by the procedures of Horecker *et al.* (1953), Smyrniotis and Horecker (1956), and Horecker (1957e). A procedure in which D-ribose 5-phosphate is 64% converted to D-sedoheptulose 7-phosphate has been described (Wood and Poon, 1970). The procedure employs a fresh rat liver supernatant as a source of isomerase, epimerase, and transketolase, and glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase (LDH) are added to drive the reaction toward completion in the presence of arsenate [Eqs. (24)–(27)].

$$\text{D-R-5-P} \overset{\text{isomerase}}{\rightleftharpoons} \text{D-Ru-5-P} \overset{\text{epimerase}}{\rightleftharpoons} \text{D-Xu-5-P} \tag{24}$$

$$\text{D-R-5-P} + \text{D-Xu-5-P} \overset{\text{TK}}{\rightleftharpoons} \text{D-S-7-P} + \text{D-GAP} \tag{25}$$

$$\text{D-GAP} + \text{arsenate} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{GAPDH}} \text{D-3-PGA} + \text{arsenate} + \text{NADH} + \text{H}^+ \tag{26}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+ \tag{27}$$

D-Sedoheptulose 7-phosphate has also been prepared by condensation of 3-hydroxypyruvate and D-ribose 5-phosphate in the presence of transketolase (Yaphe *et al.*, 1966). Sedoheptulose 7-phosphate labeled with ^{14}C in the

1-, 2-, and 3-positions has been prepared from labeled D-fructose 6-phosphate and unlabeled D-erythrose 4-phosphate using transaldolase (Eidels and Osborn, 1974).

Properties

Treatment with 1 *N* sulfuric acid at 100°C for 20 min split off 11% of the phosphate (Bucolo and Bartlett, 1960). Its lability in acid resembles that of fructose 6-phosphate 20% being hydrolyzed by 1 *N* sulfuric acid at 100°C in 2 hr (Horecker, 1957e). At a 0.1 *M* concentration it reacts with phosphofructokinase at about one-tenth the rate at which it reacts with fructose 6-phosphate, but at 2 m*M* the two rates are approximately equal (Wood, 1975a).

Analysis

Dische (1953) described three different variations of the reaction with orcinol and ferric chloride for the colorimetric determination of heptoses and a reaction with cysteine in the presence of concentrated sulfuric acid, which has been widely used. Heptuloses may be estimated in the presence of pentoses by using the absorbance values at 580 and 670 nm in the orcinol reaction (Horecker, 1957e). The orcinol–ferric chloride reaction of Dische was used to measure the production of sedoheptulose 7-phosphate from ribose 5-phosphate by hemolysates (Bruns *et al.*, 1958a), and some further improvements were suggested by Novello and McLean (1968). A comparison of the cysteine–sulfuric acid colorimetric methods with an enzymatic assay and the effect of added sugar phosphates and arsenate has been made. Sedoheptulose 7-phosphate appeared to give about 25% more color in the cysteine–sulfuric acid reaction than an equivalent amount of sedoheptulosan (Wood and Poon, 1970).

An enzymatic assay [Eqs. (28)–(30)] using transaldolase was introduced by Cooper *et al.* (1958).

$$\text{D-F-1,6-PP} \xrightleftharpoons{\text{aldolase}} \text{DHAP} + \text{D-GAP} \tag{28}$$

$$\text{D-S-7-P} + \text{D-GAP} \xrightleftharpoons{\text{TA}} \text{D-Er-4-P} + \text{D-F-6-P} \tag{29}$$

$$\text{D-F-6-P} + \text{NADP}^+ + \text{H}_2\text{O} \xrightarrow{\text{PGI/G-6-PDH}} \text{D-6-PG} + \text{NADPH} + \text{H}^+ \tag{30}$$

This assay was found to be sluggish and slow to reach completion, and an assay using phosphofructokinase and aldolase was introduced [Eqs. (31)–(33)].

$$\text{D-S-7-P} + \text{ATP} \xrightarrow{\text{phosphofructokinase}} \text{D-S-1,7-PP} + \text{ADP} \tag{31}$$

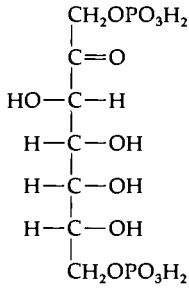
$$\text{D-S-1,7-PP} \xrightleftharpoons{\text{aldolase}} \text{D-Er-4-P} + \text{DHAP} \quad (32)$$

$$\text{DHAP} + \text{NADH} + \text{H}^+ \xrightarrow{\text{GlPDH}} \text{GlP} + \text{NAD}^+ \quad (33)$$

This assay is much more rapid, but any fructose 6-phosphate and fructose or sedoheptulose bisphosphates present must be determined and subtracted from the result (Wood and Poon, 1970; Wood, 1975a).

An enzymatic assay involving the oxidation of the sedoheptulose 7-phosphate–transketolase complex with ferricyanide has been described by Kochetov (1982d).

D-Sedoheptulose 1,7-Bisphosphate



Preparation

The preparation of D-sedoheptulose 1,7-bisphosphate in admixture with other sugar phosphates has been described (Horecker *et al.*, 1955; Smyrniotis and Horecker, 1956; Bucolo and Bartlett, 1960). However, improved methods are now available, capable of giving yields of the desired product approaching 100%. The first procedure condenses D-erythrose 4-phosphate with dihydroxyacetone phosphate in the presence of aldolase [Eqs. (34)–(36)] (Tsolas, 1975).

$$\text{D-F-1,6-PP} \xrightleftharpoons{\text{aldolase}} \text{D-GAP} + \text{DHAP} \quad (34)$$

$$\text{D-GAP} \xrightleftharpoons{\text{TPI}} \text{DHAP} \quad (35)$$

$$\text{D-Er-4-P} + \text{DHAP} \xrightleftharpoons{\text{aldolase}} \text{D-S-1,7-PP} \quad (36)$$

The second procedure uses phosphofructokinase to phosphorylate sedoheptulose 7-phosphate in the 1-position [Eq. (37)] (Wood and Poon, 1970; Paoletti *et al.*, 1979b).

$$\text{D-S-7-P} + \text{ATP} \xrightarrow{\text{phosphofructokinase}} \text{D-S-1,7-PP} + \text{ADP} \quad (37)$$

The bisphosphate labeled on C-1, C-2, and C-3 has been produced by incubating it with ^{14}C fructose bisphosphate and aldolase (Smyrniotis and Horecker, 1956) and ^{32}P has been attached to C-1 by using [γ-^{32}P]ATP and phosphofructokinase (Paoletti *et al.*, 1979b).

Properties

Treatment with 1 *N* sulfuric acid at 100°C split off 50% of the total organic phosphate in 20–30 min, at the same rate as the phosphate was removed from sedoheptulose 1-phosphate (Horecker, 1957e; Bucolo and Barlett, 1960). The remaining phosphate was cleaved from the 7-position at the same rate as sedoheptulose 7-phosphate was split (Bucolo and Bartlett, 1960). The specific rotation at 5461 Å = +8° (Robison *et al.*, 1938). It is hydrolyzed to inorganic phosphate and the 7-phosphate by a specific bisphosphatase, which has been purified from yeast (Racker and Schroeder, 1958; Racker, 1962a). It is cleaved by muscle aldolase (K_m = 0.1 m*M*) at approximately 60% of the rate at which fructose 1,6-bisphosphate (K_m = 0.06 m*M*) is attacked (Horecker, 1957e), and it has been suggested that it serves as a biological reservoir of erythrose 4-phosphate (Horecker *et al.*, 1955).

Analysis

D-Sedoheptulose 1,7-bisphosphate may be measured colorimetrically using the cysteine–sulfuric acid reaction (Paoletti *et al.*, 1979b). Following cleavage by aldolase as described previously, the dihydroxyacetone phosphate formed may be measured enzymatically using NADH and glycerophosphate dehydrogenase, or the second product, erythrose 4-phosphate, may be measured using the specific assay for that compound (Paoletti *et al.*, 1979c) or with transaldolase [Racker and Schroeder, 1958; Eqs. (38)–(41)].

$$\text{D-S-1,7-PP} \xrightleftharpoons{\text{aldolase}} \text{D-Er-4-P} + \text{DHAP} \quad (38)$$

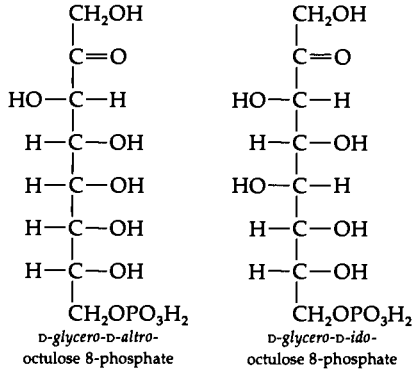
$$\text{DHAP} + \text{NADH} + \text{H}^+ \xrightarrow{\text{GlPDH}} \text{GlP} + \text{NAD}^+ \quad (39)$$

$$\text{D-Er-4-P} + \text{D-F-6-P} \xrightleftharpoons{\text{TA}} \text{D-S-7-P} + \text{D-GAP} \quad (40)$$

$$\text{D-GAP} + \text{NADH} + \text{H}^+ \xrightarrow{\text{TPI/GlPDH}} \text{GlP} + \text{NAD}^+ \quad (41)$$

Racker and Schroeder (1958) also used the specific sedoheptulose bisphosphatase to hydrolyze it and measured the inorganic phosphate produced.

Octulose Phosphates



The formation of an octulose 8-phosphate by transaldolase acting on ribose 5-phosphate and fructose 6-phosphate as substrates has been observed (Racker and Schroeder, 1957; Bonsignore *et al.*, 1959). Small amounts of sedoheptulose 7-phosphate, the 1,7-bisphosphate, an octulose 1-phosphate, and a 1,8-bisphosphate were reported in hemolysates of human erythrocytes incubated with inosine and inorganic phosphate (Bucolo and Bartlett, 1960; Bartlett and Bucolo, 1960, 1968). Similarly, Vanderheiden (1964, 1965) found sedoheptulose 1,7-bisphosphate and octulose 1,8-bisphosphate in normal red cells. The bisphosphates were believed to have resulted from aldolase condensation of dihydroxyacetone phosphate with D-erythrose 4-phosphate and D-ribose 5-phosphate and the octulose 1-phosphate by reaction with free ribose (Bartlett and Bucolo, 1960, 1968). Paoletti *et al.* (1979b) have identified in rat liver small amounts of D-sedoheptulose 1,7-bisphosphate, D-*glycero*-D-*altro*-octulose 8-phosphate, D-*glycero*-D-*ido*-octulose 8-phosphate, and the corresponding 1,8-bisphosphates and have suggested that they may be intermediates in an expanded version of the pentose phosphate pathway (see Chapter 2) rather than due incidentally to the presence of aldolase in the tissue.

The D-*glycero*-D-*altro*- and the D-*glycero*-D-*ido*-octulose 1,8-bisphosphates were synthesized as already described by aldolase condensation of dihydroxyacetone phosphate with D-ribose 5-phosphate and D-arabinose 5-phosphate, respectively (Paoletti *et al.*, 1979a). It has been shown that none of the D-*glycero*-D-*altro*-octulose 1,8-bisphosphate was present in solution in the free keto form (Gray and Barker, 1970). The bisphosphates yielded 50% of their total phosphate as inorganic phosphate on treatment with 1 *N* hydrochloric acid for 15 min at 100°C. They were not acted on by liver fructose bisphosphatase and gave characteristic colors in the cysteine–

sulfuric acid reaction. The kinetic constants for their synthesis and cleavage by liver and muscle aldolases were also determined (Paoletti *et al.*, 1979a). Octulose 8-phosphate, presumably the D-*glycero*-D-*altro*-compound, was 15% hydrolyzed by 1 *N* hydrochloric acid in 15 min at 100°C (Racker and Schroeder, 1957).

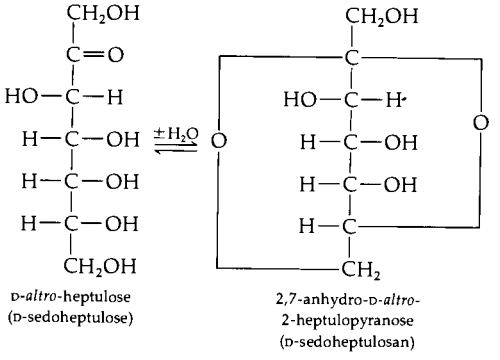
D-Ribulose

The sugar may be obtained by refluxing D-arabinose (Cohen, 1953) or D-ribose in dry pyridine or, alternatively, by oxidation of ribitol using ribitol dehydrogenase (Bruns *et al.*, 1958b; Mortlock and Wood, 1966) or whole cells of *Klebsiella aerogenes* (Mortlock, 1975). Storage of solutions of the sugar led to a slow isomerization to D-arabinose (Cohen, 1953). The specific rotation of D-ribulose is −15° (Hickman and Ashwell, 1956); the specific rotation of the crystalline *ortho*-nitrophenylhydrazone is −52 ± 5°, and the melting point is 165–166.5°C (Horecker *et al.*, 1951; Cohen, 1953).

D-Xylulose

D-Xylulose may be prepared by refluxing D-xylose in dry pyridine or by oxidizing D-arabitol with D-arabitol dehydrogenase (Mortlock and Wood, 1966) or with whole cells of *Acetobacter suboxydans* (Stumpf and Horecker, 1956). The specific rotation has been reported as −33.2° (Ashwell and Hickman, 1954; Hickman and Ashwell, 1956) and −36.8° (Hurwitz and Horecker, 1956). The melting point of the crystalline *para*-bromophenylhydrazone was 126–129°C (Hochster, 1955; Hickman and Ashwell, 1956). An enzymatic assay using D-xylulose kinase has been described (Stumpf and Horecker, 1956; Hurwitz and Horecker, 1956).

D-Sedoheptulose and D-Sedoheptulosan



D-*altro*-heptulose (D-sedoheptulose)

2,7-anhydro-D-*altro*-2-heptulopyranose (D-sedoheptulosan)

D-Sedoheptulosan is commercially available; when a solution in 3 *N* hydrochloric acid is kept for 12 hr at room temperature, an equilibrium mixture of 20% D-sedoheptulose and 80% D-sedoheptulosan is obtained (Ujejski and Waygood, 1955). D-Sedoheptulosan is stable to alkali and forms a hydrate, melting point = 101–102°C; specific rotation = −134°, or −146° calculated as the anhydrous heptulosan (Pratt *et al.*, 1952).

4

Preparation, Properties, and Analysis of the Enzymes of the Pathway

INTRODUCTION

The two dehydrogenases have been discussed in detail by Noltmann and Kuby (1963) and the glucose 6-phosphate dehydrogenase of red blood cells by Marks (1964). Information may also be found in Barman (1969), in Bergmeyer (1974), in the article by Waygood and Rohringer (1964), and in monographs specifically oriented toward the dehydrogenases. This chapter is intended to provide information on the four enzymes of the nonoxidative branch that will supplement information already published in the series entitled "The Enzymes."

D-RIBOSE-5-PHOSPHATE KETOL-ISOMERASE (EC 5.3.1.6)

D-ribose 5-phosphate ⇌ D-ribulose 5-phosphate

D-Ribose-5-phosphate ketol-isomerase was discussed very briefly in Volume 5 of the second edition of "The Enzymes" (Topper, 1961) and in more detail in Volume 6 of the third edition (Noltmann, 1972).

Purification

The enzyme has been purified from a wide range of sources as summarized in Table 4.1. The methods employed include ammonium sulfate fractionation, heat denaturation, chromatography on ion exchangers, gel filtration, preparative electrophoresis, and affinity chromatography on

TABLE 4.1

Purification of Ribose-5-Phosphate Ketol-Isomerase from Various Sources

Source	Reference
Alfalfa	Axelrod and Jang (1954)
Spinach	Tabachnik *et al.* (1958)
Spinach	Rutner (1970)
Spinach	Knowles *et al.* (1980)
Spinach	Hurwitz *et al.* (1956)
Spinach	Ivanishchev *et al.* (1982)
Pea leaf	Anderson (1971)
Candida utilis	Domagk *et al.* (1973), Domagk and Doering (1975)
Candida utilis	Horitsu and Tomoyeda (1966), Horitsu *et al.* (1976, 1979)
Rhodospirillum rubrum	Anderson and Fuller (1968)
Echinococcus granulosus	Agosin and Aravena (1960b)
Pediococcus pentosaceus	Dobrogosz and DeMoss (1963)
Aerobacter aerogenes	Matsushima and Simpson (1965)
Escherichia coli K12	Essenberg and Cooper (1975)
Escherichia coli	David and Wiesmeyer (1970b)
Human erythrocytes	Urivetzky and Tsuboi (1963)
Rabbit muscle	Dickens and Williamson (1956)
Lingcod muscle	Tarr (1959)
Ox muscle, calf spleen and liver	Kiely *et al.* (1973)
Ox muscle	Domagk *et al.* (1974), Domagk and Alexander (1975)
Rabbit muscle	Knowles *et al.* (1980)
Thiobacillus thioparus	Middaugh and MacElroy (1976), MacElroy and Middaugh (1982)
Bacillus caldolyticus	Middaugh and MacElroy (1976), MacElroy and Middaugh (1982)

TABLE 4.2

Specific Activities of Purified Ribose-5-Phosphate Ketol-Isomerase from Various Sources

Specific activity (units/mg)	Source	Reference
13	*Pediococcus pentosaceous*	Dobrogosz and DeMoss (1963)
20	Lingcod muscle	Tarr (1959)
356(h)[a]	*Candida utilis*	Domagk *et al.* (1973)
33(h)	*Candida utilis*	Horitsu *et al.* (1978)
38	Human erythrocytes	Urivetzky and Tsuboi (1963)
63	Calf spleen	Kiely *et al.* (1973)
49	Ox muscle	Kiely *et al.* (1973)
355(h)	Ox muscle	Domagk *et al.* (1974)
53	*Bacillus caldolyticus*	MacElroy and Middaugh (1982)
260	Hydatid cysts	Agosin and Aravena (1960b)
544	*Thiobacillus thioparus*	MacElroy and Middaugh (1982)
2171(h)	Spinach	Rutner (1970)
2400	Alfalfa	Axelrod and Jang (1954)
2500	Spinach	Knowles *et al.* (1980)
76(h)	Spinach	Ivanishchev *et al.* (1982)

[a] h, claimed to be homogeneous.

para-mercuribenzoate-6-aminohexyl-Sepharose 4B (Horitsu *et al.*, 1979). The specific activities of the purified enzyme from various sources are given in Table 4.2.

Stability

In general, the enzyme is stable and quite resistant to heat, and a heating step has been used by several workers to remove nonenzyme protein. However, the *Lactobacillus plantarum* enzyme was destroyed by heat (Hurwitz and Horecker, 1956). Matsushima and Simpson (1965) reported that the enzyme from *Aerobacter aerogenes* lost activity on storage, which could be restored by cysteine. Rutner (1970) included EDTA and mercaptoethanol in all his buffers in order to stabilize the enzyme, and the enzyme from *Candida utilis* was reported to be unstable in dilute solution (Horitsu *et al.* 1976). The stability of the enzymes from a mesophilic bacterium and from a thermophile were investigated by Middaugh and MacElroy (1976), who found them to be freeze labile, stabilized by certain salts, and destabilized by lithium bromide, calcium chloride, and organic solvents. The alfalfa enzyme had maximal stability in the pH range 4–8 (Axelrod and Jang, 1954), and the enzyme from *Aerobacter aerogenes* was best stored at pH 7.5 (Matsushima and Simpson, 1965).

Assay Methods

Nearly all the assay methods follow the forward reaction in which ribose 5-phosphate is converted to ribulose 5-phosphate. The most common assay is that introduced by Axelrod and Jang (1954) in which the ribulose 5-phosphate formed is measured using the cysteine–carbazole reaction. The disappearance of ribose 5-phosphate may also be measured using the reaction with phloroglucinol (Ashwell and Hickman, 1957; Bruns *et al.*, 1958). The change in optical rotation of the reaction mixture was used by Tabachnik *et al.*, (1958) to follow the reaction. They also added an excess of epimerase, stopped the reaction, and measured the xylulose 5-phosphate formed with transketolase. They showed that the amount of xylulose 5-phosphate was proportional to the amount of isomerase added to the reaction mixture. The assay was converted into a continuous spectrophotometric procedure by Novello and McLean (1968), who added NADH, an excess of epimerase, an excess of transketolase, glycerophosphate dehydrogenase, and triose-phosphate isomerase. Production of ribulose 5-phosphate led to an increase in absorbance at 340 nm (Scheme 1). A similar procedure was used by Yaphe *et al.*, (1966), who, however, used ribulose 5-phosphate and xylulose 5-phosphate as the substrates and measured the rate of the reverse reaction (Scheme 2). A coupled reaction in which epimerase and phosphoketolase were used to determine the ribulose 5-phosphate formed was used by David and Wiesmeyer (1970a) (Scheme 3).

The discovery that the product of the action of isomerase on D-ribose 5-phosphate had appreciable absorbance in the ultraviolet region (Knowles and Pon, 1968) led to the development of direct spectrophotometric assays for the enzyme, one at 280 nm (Knowles *et al.*, 1969), the other at 290 nm (Wood, 1970; Wood, 1975c). Assays of the enzyme in a variety of rat tissues by the 290 nm method and by a coupled assay at 340 nm have been compared (Wood, 1974b). A coupled reaction using bicarbonate, NADPH, and 6-phosphogluconate dehydrogenase has been described by Yadava and Ross (1975) (Scheme 4).

The enzyme reaction was followed in the reverse direction by Kiely *et al.*, (1973). They used ribulose 5-phosphate as the substrate and the cysteine–carbazole reaction to measure its disappearance, and they determined the

$$\begin{aligned} \text{R—5—P} &\rightleftharpoons \text{Ru—5—P} \\ \text{Ru—5—P} &\rightleftharpoons \text{Xu—5—P} \\ \text{R—5—P + Xu—5—P} &\rightleftharpoons \text{S—7—P + GAP} \\ \text{GAP} &\rightleftharpoons \text{DHAP} \\ \text{DHAP + NADH + H}^+ &\rightarrow \text{GlP + NAD}^+ \end{aligned}$$

Scheme 1. Assay of ribose-5-phosphate ketol-isomerase with D-ribose 5-phosphate as substrate.

$$\text{Ru—5—P} \rightleftharpoons \text{R—5—P}$$
$$\text{Xu—5—P} + \text{R—5—P} \rightleftharpoons \text{S—7—P} + \text{GAP}$$
$$\text{GAP} + \text{NAD}^+ + \text{arsenate} \rightarrow \text{3—PGA} + \text{NADH} + \text{H}^+$$

Scheme 2. Assay of ribose-5-phosphate ketol-isomerase with D-ribulose 5-phosphate and D-xylulose 5-phosphate as substrates.

Michaelis constant of D-ribulose 5-phosphate (Table 4.6). The coupled assay for the reverse reaction has already been described (Yaphe *et al.*, 1966) (Scheme 2).

Isozymes

The existence of isozymes of isomerase was first reported by David and Wiesmeyer (1970b), who found two types of activity in *Escherichia coli*, one was heat labile and the other heat stable. Skinner and Cooper (1971) suggested that two forms were present in *E. coli K12* from their finding of two different K_m values (Table 4.6), and Essenberg and Cooper (1975) showed that the high-K_m enzyme was a constitutive enzyme and the low-K_m enzyme was an inducible enzyme. It was suggested that each isozyme is adapted specifically to catalyze the isomerase reaction in either the forward or the reverse direction (David and Wiesmeyer, 1970b; Skinner and Cooper, 1971). Anderson (1971) reported the presence of two isozymes in pea leaf, one located in the chloroplasts and the other in the cytoplasm. The K_m values, inhibitor constants, and optimal pH were almost identical, and the only definite difference between the two was in the isoelectric points (4.95 for the chloroplast enzyme and 4.75 for the enzyme in the cytoplasm). In spinach, Rutner (1970) could find no evidence for the presence of more than one form of isomerase. However, his value for the K_m was only one-tenth that reported by Knowles *et al.*, (1969) (Table 4.6). In later work, two enzyme forms were reported in spinach, the major isozyme being located within the chloroplast and the minor isozyme, presumably in the cytoplasm (Knowles *et al.*, 1980).

In 1974, a specific enzyme stain for isomerase was developed (Wood, 1974b) and it was shown by agar gel electrophoresis that three forms of the enzyme were present in spinach and two forms in certain rat tissues. The

$$\text{R—5—P} \rightleftharpoons \text{Ru—5—P}$$
$$\text{Ru—5—P} \rightleftharpoons \text{Xu—5—P}$$
$$\text{Xu—5—P} + \text{P}_i \rightleftharpoons \text{acetyl phosphate} + \text{GAP (phosphoketolase)}$$
$$\text{GAP} \rightleftharpoons \text{DHAP}$$
$$\text{DHAP} + \text{NADH} + \text{H}^+ \rightarrow \text{GlP} + \text{NAD}^+$$

Scheme 3. Assay of ribose-5-phosphate ketol-isomerase using phosphoketolase.

$$R—5—P \rightleftharpoons Ru—5—P$$
$$Ru—5—P + CO_2 + NADPH + H^+ \rightarrow 6\text{-phosphogluconate} + NADP^+$$

Scheme 4. Assay of ribose-5-phosphate ketol-isomerase using NADPH, 6-phosphogluconate dehydrogenase, and bicarbonate.

two forms in rat spleen were partially separated and compared with each other and the single form in rat liver. No significant differences in the Michaelis Constants could be detected (Table 4.6). Using column chromatography, the existence of more than one form of the enzyme in rat spleen was confirmed by Glushankov and Ipatova (1976).

Starch-gel electrophoresis has been used to search for isozymes in human tissues using exogenous coupling enzymes to reveal isomerase activity. Only one molecular species was present in the 10 tissues examined, but in a man-mouse somatic cell hybrid an additional species, attributed to the presence of a hybrid isozyme, was detected (Spencer and Hopkinson, 1980).

Three isozymes have been purified from *Candida utilis* and shown to differ in their pI and K_m values and energies of activation (Table 4.3) (Horitsu *et al.*, 1978). On the basis of its activation energy and K_m value, the enzyme isolated previously (Horitsu *et al.*, 1976) appeared to correspond to isozyme III.

Molecular Weight and Subunit Structure

Values for the molecular weight and the subunit composition are listed in Table 4.4. Whenever the subunit structure has been investigated, isomerase has been found to be made up of two or more subunits of molecular weight, 26,000 or some multiple of this figure. Isomerase isolated from *Candida utilis* by Horitsu *et al.* (1976) is noteworthy for being a trimer. The discrepancy between their results and those of Doering *et al.* (1973), who also isolated the enzyme from *Candida utilis*, was attributed by the Japanese workers to differences in the strain of yeast used.

TABLE 4.3

Properties of Isomerase Isozymes from Candida utilis[a]

Form	pI	K_m (m*M*)	Activation energy (cal/mole)
I	3.79	0.77	10,800
II	3.87	0.67	12,000
III	3.99	0.16	6,360

[a] From Horitsu *et al.* (1978).

TABLE 4.4

Molecular Weights and Subunit Structure of Isomerase from Various Sources

Source	Molecular weight	Subunits	Reference
Spinach	53,200	2 × 26,000	Rutner, (1970), Noltmann (1972), Ivanishchev *et al.* (1982)
Rhodospirillum rubrum	57,000	—	Anderson *et al.* (1968)
Chromatium D	54,000	—	Gibson and Hart (1969)
Candida utilis	105,000	4 × 26,000	Doering *et al.* (1973)
Candida utilis	183,000	1 × 75,000 & 2 × 54,000	Horitsu *et al.* (1976)
Escherichia coli I[a]	45,000	—	Essenberg and Cooper (1975)
Escherichia coli II[a]	33,000	—	Essenberg and Cooper (1975)
Bacillus caldolyticus	40,000	—	Middaugh and MacElroy (1976)
Thiobacillus neapolitans	40,000	—	Middaugh and MacElroy (1976)
Beef muscle	228,000	4 × 58,000	Domagk *et al.* (1974)

[a] I, constitutive enzyme; II, inducible enzyme.

Electrophoresis and Isoelectric Point

The beef muscle enzyme had a pI equal to 5.9 (Domagk et al., 1974) and that from *Candida utilis* a value of 4.7 (Domagk et al., 1973). The three forms isolated from *Candida utilis* by the Japanese workers had pI values of 3.79, 3.87, and 3.99 (Horitsu et al., 1978). The two isozymes in pea leaf had values of 4.75 and 4.95 (Anderson, 1971).

Equilibrium of the Isomerase Reaction

The values reported by various workers are listed in Table 4.5. If we ignore the extreme values obtained by the earlier workers, Tarr (1959) and Urivetzky and Tsuboi (1963), at pH 7.5 and 37°C, most workers have obtained a value close to that of Axelrod and Jang (1954). As the temperature decreases, less ribulose 5-phosphate is present at equilibrium. Standard changes in the thermodynamic parameters for the reaction have been reported as $\Delta G = +700$ cal/mole, $\Delta H = -3060$ cal/mole, and $\Delta S = -12.1$ e.u. (Axelrod and Jang, 1954); $\Delta G = +717$ cal/mole, $\Delta H = +7804$ cal/mole, and $\Delta S = -22.4$ e.u. (Agosin and Aravena, 1960b); $\Delta G = -5629$ cal/mole, $\Delta H = +4336$ cal/mole, and $\Delta S = +32.15$ e.u. (Horitsu *et al.*, 1976).

TABLE 4.5

Values of the Equilibrium Constant for the Isomerase Reaction

$$K_{eq} = \frac{\text{D-ribose 5-phosphate}}{\text{D-ribulose 5-phosphate}}$$

K_{eq}	Conditions	Reference
0.82	60°C	Hurwitz *et al.* (1956)
1.35	RT	Tarr (1959)
2.57	37°C, pH 7.5	Urivetzky and Tsuboi (1963)
3.00	RT	Tabachnik *et al.* (1958)
3.17	30°C, pH 7.5	Bruns *et al.* (1958b)
3.21	38°C	Agosin and Aravena (1960b)
3.33	37°C	Dobrogosz and DeMoss (1963)
3.10	37°C, pH 7.0	Axelrod and Jang (1954)
3.79	25°C, pH 7.0	Axelrod and Jang (1954)
6.10	0°C, pH 7.0	Axelrod and Jang (1954)

Kinetic Properties

With the exception of Agosin and Aravena (1960b), who reported isomerase from *Echinococcus granulosus* to be most active in the range pH 6.0 to 7.5, all other workers agree that the optimal pH range is in the region from 7.0 to 8.5 (Axelrod and Jang, 1954; Dickens and Williamson, 1956; Bruns *et al.*, 1958b; Tarr, 1959; Urivetzky and Tsuboi, 1963; Matsushima and Simpson, 1965; Horitsu and Tomoyeda, 1966; Novello and McLean, 1968; Anderson, 1971).

Except for Kiely *et al.* (1973), all workers who have studied the kinetic properties have measured the Michaelis constant of the enzyme for D-ribose 5-phosphate (Table 4.6). Kiely *et al.* determined the K_m of spinach isomerase for D-ribulose 5-phosphate by measuring its rate of disappearance directly and also calculated it using the Haldane relationship. The values for the K_m of D-ribose 5-phosphate determined for the spinach enzyme by various workers are all in the region 2.0–5.4 m*M*, apart from the exceptionally low value obtained by Rutner (1970). Noltmann (1972) has suggested that this lack of agreement may either be of methodological origin or be due to the transformation of a "low-K_m" enzyme into a "high-K_m" enzyme by the heat-treatment step in the purification.

Activation energies for the forward reaction have been determined as 8900 cal/mole (Bruns *et al.*, 1958b) for the red blood cell enzyme and values of 10,800, 12,000, and 6,360 cal/mole for the three enzymes from *Candida*

TABLE 4.6

Michaelis Constants of Ribose-5-Phosphate Isomerase

K_m[a] (m*M*)	Source	Reference
4.6	Spinach	Knowles *et al.* (1969)
0.46	Spinach	Rutner (1970)
5.4	Spinach	Kiely *et al.* (1973)
0.78*	Spinach	Kiely *et al.* (1973)
3.1[b]	Spinach	Yadava and Ross (1975)
1.3[b]	Spinach	Yadava and Ross (1975)
2.0	Spinach	Ivanishchev (1982)
3.3	Spinach	Woodruff and Wolfenden (1979)
2.1, 2.3	Pea leaf	Anderson (1971)
2.5	*Candida utilis*	Domagk *et al.* (1973)
0.16, 0.67, 0.77	*Candida utilis*	Horitsu *et al.* (1978)
1.8	*Aerobacter aerogenes*	Matsushima and Simpson (1965)
2.7	*Echinococcus granulosus*	Agosin and Aravena (1960b)
2.8	*Pediococcus pentosaceous*	Dobrogosz and DeMoss (1963)
0.43	*Rhodospirillum rubrum*	Anderson and Fuller (1968)
0.19, 5.4	*Escherichia coli*	David and Wiesmeyer (1970b)
0.95, 6.2	*Escherichia coli K12*	Skinner and Cooper (1971)
0.83, 4.4	*Escherichia coli*	Essenberg and Cooper (1975)
0.32	Rat liver	Wood (1974b)
0.33	Rat spleen	Wood (1974b)
4.9	Rat brain	Kauffman (1972)
1.0	Bovine lens	Srivastava and Devi (1967)
2.6	Calf spleen	Kiely *et al.* (1973)
9.6	Calf liver	Kiely *et al.* (1973)
6.5	Ox muscle	Kiely *et al.* (1973)
2.0	Ox muscle	Domagk and Alexander (1975)
2.1	Human erythrocytes	Urivetzky and Tsuboi (1963)

[a] All the values listed are for D-ribose 5-phosphate as substrate except the one marked with an asterisk, which is for D-ribulose 5-phosphate. Where more than one value is listed, the values refer to different (isoenzymic) forms of the enzyme.

[b] Lower value by cysteine–carbazole assay, higher value by a new spectrophotometric assay.

utilis (Horitsu *et al.*, 1978). Middaugh and MacElroy (1976) found sharp discontinuities in Arrhenius plots of K_m and V_{max} for the isomerases from a mesophilic and a thermophilic bacterium at temperatures characteristic of the milieu in which they operate. These were interpreted as due to a temperature-dependent change in enzyme conformation.

Inhibition

Sulfhydryl Reagents

The enzyme from most sources has been reported to be inhibited by compounds reacting with thiol groups, such as *para*-chlormercuribenzoate and *N*-ethylmaleimide, and by heavy metals, for example, the enzymes from red cells (Bruns, *et al.*, 1958b; Urivetzky and Tsuboi, 1963), from spinach (Tabachnik *et al.*, 1958; Rutner, 1970), from *Aerobacter aerogenes* (Matsushima and Simpson, 1965), from *Echinococcus granulosus* (Agosin and Aravena, 1960b), from beef muscle (Domagk *et al.*, 1974), and from *Candida utilis* (Doering *et al.*, 1973). However, despite their earlier report (Horitsu and Tomoyeda, 1966) that the enzyme from *Candida utilis* was inhibited by *para*-chlormercuribenzoate and iodoacetate, it was later stated that the three isozymes from this yeast were unusual in not being affected by sulfhydryl reagents (Horitsu *et al.*, 1978).

Iodoacetate and iodoacetamide were somewhat more selective. There was no inhibition of the red cell enzyme (Bruns *et al.*, 1958b; Urivetzky and Tsuboi, 1963) by iodoacetate, and Essenberg and Cooper (1975) reported that the inducible enzyme from *E. coli* was inhibited by 1.25 m*M* iodoacetate, while the constitutive enzyme was not.

It was reported that activity lost by the *Aerobacter aerogenes* enzyme on storage could be restored by the addition of cysteine, glutathione, or mercaptoethanol (Matsushima and Simpson, 1965), and MacElroy and Middaugh (1982) have reported stimulation of the enzyme by mercaptoethanol.

EDTA and Metal Ions

EDTA had no inhibitory effect on the enzymes from *Echinococcus granulosus* (Agosin and Aravena, 1960b) or red blood cells (Bruns *et al.*, 1958b), and it was reported to stimulate the enzymes from *Bacillus caldolyticus* and *Thiobacillus thioparus* (MacElroy and Middaugh, 1982). By contrast, the enzymes from *Candida utilis* appeared to be metallo-enzymes, being inhibited by EDTA and activated by Co^{2+} and Mg^{2+} (Horitsu and Tomoyeda, 1966, Horitsu *et al.*, 1978). The *Echinococcus granulosus* enzyme has been reported to be stimulated by 17 m*M* sodium or potassium ion (Agosin and Aravena, 1960b).

Phosphorus Compounds

Inhibition by relatively high concentrations of *ortho*-phosphate has been reported by Axelrod and Jang (1954), Matsushima and Simpson (1965), Rutner (1970), and Anderson and Fuller (1968), who found the inhibition was competitive with ribose 5-phosphate with a K_i value for the enzyme

from *Rhodospirillum rubrum* of 67 ± 30 m*M*. Pyrophosphate was found to inhibit at concentrations around 1 m*M* (Horitsu and Tomoyeda, 1966). Strong inhibition by 0.13 m*M* 5-phosphoribonate was reported by Axelrod and Jang (1954), and a K_i of 0.119 m*M* has been measured for the spinach enzyme (Woodruff and Wolfenden, 1979), but Tarr (1959) found only 45% inhibition by a 5 m*M* concentration. A very strong inhibition by the transition-state analog D-4-phosphoerythronic acid has been reported (K_i = 4.4 μM; Woodruff and Wolfenden, 1979). 6-Phosphogluconate inhibited at fairly high concentrations (Dobrogosz and MeMoss, 1963; Mutsushima and Simpson, 1965; David and Wiesmeyer, 1970b), but a low K_i value of 0.61 m*M* was determined by Japanese workers for their enzyme from *Candida utilis* (Horitsu *et al.*, 1976).

D-Erythrose 4-phosphate has been reported to be a moderately strong inhibitor effective at concentrations around 1 m*M* (Matsushima and Simpson, 1965; Woodruff and Wolfenden, 1979), and the triose phosphates (Agosin and Arevena, 1960b) and hexose phosphates (Axelrod and Jang, 1954; David and Wiesmeyer, 1970b) have also been identified as inhibitors.

A number of authors have studied inhibition by adenine nucleotides (Axelrod and Jang, 1954; Anderson and Fuller, 1968; David and Wiesmeyer, 1970b; Anderson, 1971) and by cytosine, guanine, and uracil mononucleotides as well (Horitsu *et al.*, 1976). Adenine and adenosine also inhibit (Axelrod and Jang, 1954). Other workers reported a lack of inhibition by adenine nucleotides (Agosin and Arevena, 1960b; Dobrogosz and DeMoss, 1963). D-Ribulose 1,5-bisphosphate was reported to be a powerful inhibitor of the enzyme from *Rhodospirillum rubrum* with a K_i of 36 μM (Anderson and Fuller, 1968).

Other Inhibitors

The following substances have also been reported as having a moderately strong inhibitory effect on isomerase: D-erythronic and D-malonic acids, D-3-phosphoglyceric acid (Woodruff and Wolfenden, 1979), D-ribose and dihydroxyacetone (DHA) (Agosin and Aravena, 1960b), and citrate (Anderson and Fuller, 1968).

Activation

Dobrogosz and DeMoss (1963) found that heat treatment of the enzyme from *Pediococcus pentosaceus* gave a 1.4-fold increase in activity, probably as the result of the denaturation of inhibitory proteins. The activity lost by the *Aerobacter aerogenes* enzyme on storage could be restored by the addition of cysteine, glutathione, or mercaptoethanol (Matsushima and Simpson, 1965), and MacElroy and Middaugh (1982) observed stimulation of two

bacterial enzymes by mercaptoethanol and by EDTA. The *Echinococcus granulosus* enzyme was stimulated by 17m*M* sodium or potassium ion (Agosin and Aravena, 1960b), and the stimulation of the *Candida utilis* enzyme by cobalt and magnesium ions has been described previously. The enzyme from the photosynthetic bacterium *Rhodospirillum rubrum* was stimulated at low concentrations of ADP and inhibited by higher concentrations (Anderson and Fuller, 1968), and in pea leaf, the enzyme appeared to be allosterically activated by D-ribulose 1,5-bisphosphate (Anderson, 1971).

Mechanism and Spectral Changes

The mechanism of aldose–ketose isomerases, in general, and of D-ribose-5-phosphate ketol-isomerase, in particular, has been discussed extensively by Topper (1961) and Noltmann (1972). The enzyme from *Candida utilis* can be inactivated by photooxidation in the presence of Rose Bengal (Doering *et al.*, 1973), and it was concluded that histidine residues played an essential role in the activity (Alexander *et al.*, 1975).

The spectral changes that accompany and follow isomerase action on D-ribose 5-phosphate have been discussed in Chapter 3 in the section on D-ribulose 5-phosphate. It has been reported that the enzyme from rabbit muscle and Baker's yeast produced only ribulose 5-phosphate, whereas that from photosynthetic sources possessed a second activity, possibly due to a contaminating enzyme, that produced 4-hydroxy-5-methyl-3(2H)-furanone (Knowles *et al.*, 1980). However, it has been shown (Wood, 1975d) that D-ribulose 5-phosphate undergoes a series of further reversible spectral changes in the absence of enzymes.

D-RIBULOSE-5-PHOSPHATE 3-EPIMERASE (EC 5.1.3.1)

D-ribulose 5-phosphate $\rightleftharpoons$ D-xylulose 5-phosphate

D-Ribulose-5-phosphate 3-epimerase was discussed briefly by Maxwell (1961) and by Glaser (1972) in "The Enzymes."

Purification

Epimerase has been purified from bacterium, yeast, and human and animal tissues (Table 4.7) and obtained in a homogeneous state from yeast (Williamson and Wood, 1966), calf liver (Wood, 1979), and human erythrocytes (Karmali *et al.*, 1983).

TABLE 4.7

Purification of D-Ribulose-5-Phosphate 3-Epimerase from Various Sources

Source	Specific activity[a] (units/mg)	Reference
Lactobacillus plantarum (L. pentosus)	69	Hurwitz and Horecker (1956), Heath *et al.* (1958), Hurwitz (1962)
Calf spleen	30	Ashwell and Hickman (1957)
Rabbit muscle	29	Tabachnik *et al.* (1958), Racker (1962b)
Lingcod	22	Tarr (1959)
Baker's yeast	258(h)	McDonough and Wood (1961), Williamson and Wood (1966)
Calf liver	617(h)	Wood (1979)
Human erythrocytes	15(h)	Karmali *et al.* (1983)

[a] h, claimed to be homogeneous

Stability

All workers with epimerase agree that it is extremely stable when stored in solution at −10 to 20 °C or in the lyophilized form. Suspensions of the calf liver enzyme in 70% saturated ammonium sulfate at 2 °C showed no loss of activity after 19 months. Both a solution and a lyophilized preparation stored at −15 °C were still active after 3 years. Repeated freezing and thawing, however, can be harmful (Wood, 1979). The enzyme from *Lactobacillus pentosus* (Stumpf and Horecker, 1956) was stable to heat, 5 min at 65 °C, and the calf liver enzyme, 2 min at 60 °C (Wood, 1979). The calf liver enzyme did not react appreciably with a 1 m*M* concentration of the protease inhibitor phenylmethylsulfonyl fluoride but was inactivated by heavy metals, and the inclusion of mercaptoethanol in the buffers used was necessary to retain its activity (Wood, 1979). The calf liver enzyme was most stable in the range pH 6.5–8.0, and a pH-stability curve has been published (Wood, 1979).

Assay Methods

A very wide range of methods has been used to assay epimerase in both the forward and the reverse direction.

Forward Reaction

Tabachnik *et al.*, (1958) incubated the enzyme with ribose 5-phosphate and excess isomerase, then they stopped the reaction and measured the xylulose 5-phosphate formed with transketolase, D-glyceraldehyde-3-phosphate dehydrogenase, NAD, and arsenate. This procedure was devel-

$$\begin{aligned} R{-}5{-}P &\rightleftharpoons Ru{-}5{-}P \\ Ru{-}5{-}P &\rightleftharpoons Xu{-}5{-}P \\ R{-}5{-}P + Xu{-}5{-}P &\rightleftharpoons S{-}7{-}P + GAP \\ GAP + \text{arsenate} + NAD^+ &\rightarrow 3{-}PGA + \text{arsenate} + NADH + H^+ \end{aligned}$$

Scheme 5. Assay of epimerase using ribose 5-phosphate, isomerase, transketolase, glyceraldehyde-3-phosphate dehydrogenase, NAD^+, and arsenate.

oped into a continuous assay by Wolin (1958). The reactions are given in Scheme 5.

The most commonly employed assay for epimerase is similar to the aforementioned reaction. It starts with ribose 5-phosphate as the substrate, but the formation of glyceraldehyde 3-phosphate is coupled to NADH oxidation via glycerophosphate dehydrogenase and triose-phosphate isomerase (Scheme 6) (Horecker *et al.*, 1956b; Novello and McLean, 1968; Tan and Wood, 1969; Kiely *et al.*, 1973; Wood, 1979).

A number of workers have used phosphoketolase to cleave the xylulose 5-phosphate formed from ribulose 5-phosphate to acetyl phosphate and glyceraldehyde phosphate (p. 50). In the original assay using phosphoketolase the [^{32}P]acetyl phosphate formed was reacted with ADP and acetokinase to give acetate and labeled ATP. The latter compound was then adsorbed onto charcoal and counted (Heath *et al.*, 1958; Hurwitz, 1962). The method was developed into a continuous assay by coupling glyceraldehyde 3-phosphate formation to NADH oxidation via triose-phosphate isomerase and glycerophosphate dehydrogenase (Scheme 7) (Williamson and Wood, 1966; David and Wiesmeyer, 1970a).

A spectrophotometric assay at 290 nm was introduced by Wood (1970, 1975c). This is based on the direct isomerase assay at 290 nm and uses ribose 5-phosphate as a substrate. The conversion of ribose 5-phosphate to ribulose 5-phosphate is allowed to reach equilibrium in the presence of an excess of isomerase. When epimerase is added, more ketopentose phosphate absorbing at 290 nm is formed and the reaction is followed in the spectrophotometer. The procedure is particularly useful for the study of inhibitors that affect transketolase and phosphoketolase but have no effect on the excess of isomerase used in the reaction (Wood, 1979). A direct assay

$$\begin{aligned} R{-}5{-}P &\rightleftharpoons Ru{-}5{-}P \\ Ru{-}5{-}P &\rightleftharpoons Xu{-}5{-}P \\ R{-}5{-}P + Xu{-}5{-}P &\rightleftharpoons S{-}7{-}P + GAP \\ GAP &\rightleftharpoons DHAP \\ DHAP + NADH + H^+ &\rightleftharpoons Glp + NAD^+ \end{aligned}$$

Scheme 6. Assay of epimerase using ribose 5-phosphate, isomerase, transketolase, glycerophosphate dehydrogenase, triose-phosphate isomerase, and NADH.

$$\text{Ru—5—P} \rightleftharpoons \text{Xu—5—P}$$
$$\text{Xu—5—P} + \text{P}_i \rightleftharpoons \text{acetyl phosphate} + \text{GAP}$$
$$\text{GAP} \rightleftharpoons \text{DHAP}$$
$$\text{DHAP} + \text{NADH} + \text{H}^+ \rightarrow \text{GlP} + \text{NAD}^+$$

Scheme 7. Assay of epimerase using ribulose 5-phosphate, phosphoketolase, glycerophosphate dehydrogenase, triose-phosphate isomerase, and NADH.

procedure that follows changes in the circular dichroism of D-ribulose 5-phosphate at 272 nm has been described by Karmali *et al.* (1983).

Reverse Reaction

The first epimerase assay that was used measured the enzyme activity in the reverse direction. Ribulose 5-phosphate formed from xylulose 5-phosphate by the enzyme was converted into ribose 5-phosphate in the presence of an excess of isomerase, and the ribose 5-phosphate was determined by the orcinol reaction (Hurwitz and Horecker, 1956). A continuous assay was used by Yaphe *et al.* (1966) in which ribose 5-phosphate and ribulose 5-phosphate were used as substrates in the presence of excess transketolase. The reactions followed either Scheme 5 or Scheme 6, according to whether the triose phosphate formed was reacted with NAD or NADH. In a similar assay developed by Wood (1974a) xylulose 5-phosphate was used as a sole substrate in the presence of an excess of isomerase, transketolase, glycerophosphate dehydrogenase, triose-phosphate isomerase, and NADH. Epimerase catalyzed the formation of ribulose 5-phosphate, which in the presence of excess isomerase furnished ribose 5-phosphate to serve as an acceptor for transketolase (Scheme 6), and it was shown that the rate of reaction was proportional to the amount of epimerase added.

Equilibrium of the Epimerase Reaction

There is a wide variation in the values determined by different groups of workers (Table 4.8). These variations are explicable by the difficulties in determining the reactant and product accurately and by a failure to attain complete equilibrium. The true value of the equilibrium constant is believed to be 3.0 at 37°C and pH 7.4.

Molecular Weight and Subunit Structure

The yeast enzyme had a molecular weight of 45,000 (Williamson and Wood, 1966). The calf liver enzyme had a molecular weight of 45,000 and appeared to consist of two identical subunits with molecular weight 22,900 (Wood, 1979). Very similar values were reported for the enzyme from

TABLE 4.8

Values for the Equilibrium Constant of the Epimerase Reaction

$$K_{eq} = \frac{\text{D-xylulose 5-phosphate}}{\text{D-ribulose 5-phosphate}}$$

K_{eq}	Conditions	Reference
1.27	37°C	Dickens and Williamson (1956)
3.0	—	Tabachnik *et al.* (1958)
0.83	37°C, pH 7.5	Stumpf and Horecker (1956)
1.5	25°C	Hurwitz and Horecker (1956)
1.4	37°C, pH 7.5	Ashwell and Hickman (1957)
3.0	37°C	Dische and Shigeura (1957)
1.5	—	Tarr (1959)
2.0	—	Lionetti and Fortier (1963)
2.4	—	Kauffman *et al.* (1969)
3.0	37°C, pH 7.4	Paoletti (1983)

human erythrocytes. This enzyme dissociated readily into its subunits in dilute solution, and the dissociation was repressed by the presence of ribulose 5-phosphate. Hill plots indicated that the binding of this substrate was cooperative (Karmali *et al.*, 1983).

Kinetic Properties

Maximal activity of epimerase is in the range pH 7–8 (Ashwell and Hickman, 1957; Williamson and Wood, 1966), and pH-activity curves have been published by Hurwitz and Horecker (1956), Tarr (1959), and Wood (1979).

Michaelis constants of 1 m*M* for D-ribulose 5-phosphate and 0.5 m*M* for D-xylulose 5-phosphate were obtained for the enzyme from *Lactobacillus plantarum* (Hurwitz and Horecker, 1956). Kiely *et al.*, (1973) obtained a K_m value for D-ribulose 5-phosphate of 2.4 m*M* for the Baker's yeast enzyme and 2.9 m*M* for the calf liver enzyme. However, later, Wood (1979) reported a value of 0.19 m*M* for the highly purified enzyme from calf liver. The discrepancy between this and the earlier value was probably due to the use of a heating step in the earlier purification procedure, which converted a "low K_m" enzyme into a "high K_m" enzyme (Noltmann, 1972). A K_m value of 0.15 m*M* D-xylulose 5-phosphate was determined for the calf liver enzyme having $K_m = 2.9$ m*M* for D-ribulose 5-phosphate (Wood, 1974a), but a value for the highly purified enzyme ($K_m = 0.19$ m*M* for D-ribulose 5-phosphate) has not been obtained. The relative rates of the forward and reverse reactions at saturating concentrations of substrate were found to be in the

ratio 30:1 (Wood, 1974a). A value of 0.143 m*M* D-ribulose 5-phosphate was determined as the apparent K_m of the enzyme in rat brain (Kauffman, 1972).

The only inhibition studies are those of Tabachnik *et al.*, (1958) and Wood (1979). Substances reacting with sulfhydryl groups were effective irreversible inhibitors. Thus, cupric and mercuric ions and *p*-chlormercuribenzoate inhibited at 1–2 m*M* following a 20-min incubation, but iodoacetamide and *N*-ethylmaleimide were only effective in the range 5–15 m*M*. 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) had no action (Wood, 1979). The rabbit muscle enzyme was 45% inhibited by 0.44 m*M* *p*-chlormercuribenzoate and 46% inhibited by incubation at 37°C for 20 min with 11 m*M* *N*-ethylmaelimide (Tabachnik *et al.*, 1958). The calf liver enzyme was reversibly inhibited 40–46% by 50 m*M* sulphate ion and 10% inhibited by 100 m*M* potassium phosphate. The substrate analogs D-glucose 6-phosphate, D-6-phosphogluconate, D-5-phosphoribonate, and D-ribose 5-phosphate had no inhibitory effect. However, D-2-deoxyribose 5-phosphate produced 45% inhibition at a concentration of 5 m*M* (Wood, 1979).

Reaction Mechanism

The reaction mechanism has been discussed by Maxwell (1961) and Glaser (1972) and is believed to proceed via the formation of an ene–diol intermediate. Studies with deuterium-labeled xylulose 5-phosphate indicate that epimerase must contain two hydrogen donor–acceptor sites, which interact with the hydrogen atom on C-3 of D-ribulose 5-phosphate and D-xylulose 5-phosphate, respectively (Davis *et al.*, 1972).

TRANSALDOLASE (EC 2.2.1.2)

D-sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate ⇌

D-fructose 6-phosphate + D-erythrose 4-phosphate

Transaldolase has been fully described in the second and third editions of "The Enzymes" (Racker, 1961b; Tsolas and Horecker, 1972) and in the "Comprehensive Biochemistry" series (Horecker, 1964). The significance of its isozymes and the mechanism of reaction have been briefly reviewed by Tsolas and Horecker (1973).

Purification

The first purification of the enzyme from a mammalian source was described by Kuhn and Brand (1972), who isolated it in a homogeneous state

from bovine mammary gland. Two other forms of the enzyme also appeared to be present. Its specific activity was only 4 units/mg compared to 48–68 units/mg for the pure yeast enzyme, but its molecular weight of 65,000 was similar. Improved procedures for the purification of isozymes I and III have been described (Schutt and Brand, 1975; Tsolas and Joris, 1975; Sun *et al.*, 1977) and their occurrence in different strains of *Candida utilis* grown under a variety of growth conditions investigated (Araujo Neto and Panek, 1979). Isozymes I and III appeared to be constitutive; the isozyme pattern was characteristic of the strain of yeast and did not change with the nutritional conditions. Isozymes I and III were coded by different genes (Sun *et al.*, 1977), while isozyme II was formed from them by hybridization (Tsolas and Horecker, 1970). It was suggested that the presence of two different isozymes in varying amounts in different strains might ensure successful survival and vigorous growth under different environmental conditions (Tsolas and Horecker, 1973).

Assay Methods

Transaldolase may be assayed in either the forward direction or the reverse direction. With sedoheptulose 7-phosphate and glyceraldehyde phosphate as substrates, the maximal rate in the forward direction is approximately one-third of the maximal rate in the reverse reaction (Wood, 1972). Assays of the enzyme in both directions have been described and the merits of the various assays discussed (Venkataraman and Racker, 1961; Wood, 1972).

When sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate are the substrates, transaldolase may be assayed according to the reactions shown in Scheme 8. These two substrates may be formed by the action of transketolase on pentose phosphates, or the sedoheptulose 7-phosphate may be placed in the reaction mixture and the glyceraldehyde 3-phosphate formed by the action of aldolase on fructose bisphosphate. Fructose 6-phosphate formation may be measured continuously in the presence of NADP, phosphoglucose isomerase, and glucose 6-phosphate dehydrogenase, or the reaction may be halted and the fructose 6-phosphate measured in a separate reaction.

The reaction in the reverse direction (Scheme 9) is faster, and inhibition of the auxiliary enzyme phosphoglucose isomerase by erythrose 4-phosphate

$$\text{S—7—P} + \text{GAP} \rightleftharpoons \text{F—6—P} + \text{Er—4—P}$$
$$\text{F—6—P} \rightleftharpoons \text{G—6—P}$$
$$\text{G—6—P} + \text{NADP} \rightarrow \text{6—PG} + \text{NADPH} + \text{H}^+$$

Scheme 8. Assay of transaldolase in the forward direction.

$$F—6—P + Er—4—P \rightleftharpoons S—7—P + GAP$$
$$GAP \rightleftharpoons DHAP$$
$$DHAP + NADH + H^+ \rightarrow GlP + NAD^+$$

Scheme 9. Assay of transaldolase in the reverse direction.

and sedoheptulose 7-phosphate is avoided. This assay is the one most generally used, and if erythrose 4-phosphate is not available, D-glyceraldehyde may be used as an acceptor to yield fructose instead of sedoheptulose 7-phosphate (Tsolas and Horecker, 1972).

Electrophoresis and Isoelectric Point

Methods for detecting activity after electrophoresis of the enzyme have been described (Tsolas and Horecker, 1970; Anderson *et al.*, 1975; Wood and Muzariri, 1981). The isoelectric points of the three isozymes from *Candida utilis* have been reported as: type I, 4.45; type II, 4.25; and type III, 3.95 (Schutt and Brand, 1975).

Kinetic Properties

Michaelis constants for fructose 6-phosphate of 0.2 m*M* and for erythrose 4-phosphate of 7 μM were measured for the enzyme from bovine mammary gland (Kuhn and Brand, 1972) and apparent K_m values of 0.055 m*M* (fructose 6-phosphate) and 4 μM (erythrose 4-phosphate) for the rat brain enzyme (Kauffman, 1972). A detailed computer analysis of the reactions catalyzed by the yeast and the bovine enzymes has been made (Kuhn and Brand, 1973; see the section on under mechanism, this chapter). The rate of the forward reaction has been measured by four different procedures and compared with that of the reverse reaction for the enzyme from *Candida utilis*. The reverse reaction had a maximal velocity three times that of the forward reaction under the conditions used (Wood, 1972).

D-Arabinose 5-phosphate was not an acceptor for yeast transaldolase but behaved as a competitive inhibitor at the acceptor site (Clark and Williams, 1971). Michaelis and inhibitor constants were measured as follows: K_m = 24 μM (D-erythrose 4-phosphate); K_i = 75 μM (D-arabinose 5-phosphate); K_m = 0.223 m*M* (D-glyceraldehyde 3-phosphate); K_i = 70 μM (D-arabinose 5-phosphate) (Williams *et al.*, 1978a). D-Erythrose 4-phosphate and D-erythrose 4-homophosphonate were both able to serve as acceptor substrates for the yeast enzyme, and the same maximal velocity was obtained as with D-erythrose 4-phosphate as the acceptor (Le Marechal *et al.*, 1980).

The transaldolase present in pea leaf chloroplasts was inactivated by light. This was taken as evidence that it functioned in the "oxidative pentose

phosphate pathway" and not in the "reductive pathway," where transaldolase is replaced by aldolase and sedoheptulose bisphosphatase (Anderson, 1981).

Reaction Mechanism

The detailed mechanism of the reaction has been discussed by Tsolas and Horecker (1972, 1973). A nonapeptide containing histidine was isolated from isozyme III following treatment with cyanogen bromide (Tsolas and Sun, 1975). As there is only one histidine residue per subunit in this isozyme, the peptide contains the histidine located at the active site, which is inactivated by photooxidation with loss of catalytic activity (Brand *et al.*, 1969). The neighbouring amino acid was cysteine, and it was deduced from models and from the fact that chlordinitrobenzene reacts with a cysteine residue to inactivate the enzyme (Rowley *et al.*, 1964) that this portion of the chain lies in a hydrophobic pocket and that the two side chains of cysteine and histidine may act in concert to stabilize a proton removed from the dihydroxyacetone intermediate. It was suggested that, although each subunit in isolation had been shown to be active (Chan *et al.*, 1973), at any given time in the intact molecule one subunit is "silent" and inactive. The corresponding nonapeptide isolated from isozyme I had the same sequence, except that a second histidine residue replaced the tyrosine residue present in the peptide from isozyme III (Schutt and Brand, 1975).

Further studies confirmed the earlier conclusion (Brand, 1970) that there were two binding sites for the donor substrate per molecule. However, the molecule bearing two dihydroxyacetone residues could not be isolated by gel filtration or stabilized by reduction with borohydride, and it appeared that the two subunits interact with strong negative cooperativity, so the only stable intermediate is the one bearing one dihydroxyacetone residue per molecule. The first subunit reacts with fructose 6-phosphate very rapidly, releasing glyceraldehyde phosphate. In the absence of an acceptor this is followed by a slow reaction with a second molecule of fructose 6-phosphate, releasing a second molecule of glyceraldehyde phosphate and a molecule of dihydroxyacetone. The rate of the fast reaction was some three orders of magnitude greater than the rate of the slow reaction (Tsolas and Horecker, 1976). The mechanism shown in Scheme 10 was suggested in which the slow reaction is the reaction of fructose 6-phosphate with the second silent subunit (Grazi *et al.*, 1977).

The enzyme isolated from bovine mammary gland, unlike the yeast enzyme, did not appear to form a transaldolase–dihydroxyacetone complex that could be isolated or fixed by borohydride reduction, and it was assumed that a different mechanism operated (Kuhn and Brand, 1972). A computer

$$\alpha\alpha + \text{F—6—P} \xrightarrow{\text{fast}} \text{DHA—}\alpha\alpha + \text{GAP}$$

$$\text{DHA—}\alpha\alpha + \text{F—6—P} \xrightarrow{\text{slow}} \text{DHA—}\alpha\alpha\text{—DHA} + \text{GAP}$$

$$\text{DHA—}\alpha\alpha\text{—DHA} \xrightarrow{\text{fast}} \text{DHA—}\alpha\alpha + \text{DHA}$$

Scheme 10. Reaction of transaldolase subunits with fructose 6-phosphate in the absence of an acceptor substrate (α, transaldolase subunit; DHA, dihydroxyacetone).

analysis of the kinetics of the yeast and bovine enzymes showed that the former reacted by a ping-pong mechanism in which a binary dihydroxyacetone–enzyme complex was formed and the latter via a ternary complex formed by a random order addition of the two substrates (Kuhn and Brand, 1973).

In a similar manner to transketolase, the carbanion intermediate formed by reaction of transaldolase with the donor substrates fructose 6-phosphate or sedoheptulose 7-phosphate can be oxidized by hexacyanoferrate to form hydroxypyruvaldehyde, which is released from the enzyme, which, in turn, is progressively inactivated during the process (Christen and Gasser, 1976; Christen *et al.*, 1976; Christen, 1977).

TRANSKETOLASE (EC 2.2.1.1)

D-xylulose 5-phosphate + D-ribose 5-phosphate $\rightleftharpoons$

D-sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate

Early studies of transketolase have been reviewed by Racker (1961a) and Horecker (1964). Further information may be found in Waygood and Rohringer (1964), Barman (1969), and Bergmeyer (1974).

Donor and Acceptor Substrates

Transketolase transfers a glycolaldehyde moiety from a suitable donor to a suitable acceptor substrate. Apart from hydroxypyruvate and dihydroxyacetone, donor substrates are all ketoses or ketose phosphates having the same configuration at C-2, C-3, and C-4, as D-xylulose (Table 4.9). Acceptor substrates, with the exception of 4-chloronitrosobenzene, are all aldehydes, and, for four-carbon and higher sugars and their phosphates, the molecules have the D-configuration at C-2 (Villafranca and Axelrod, 1971). Since D-deoxyribose 5-phosphate bears two hydrogen atoms on C-2, it also fits into this category. The lower aldehydes, formaldehyde, glycolaldehyde (with two hydrogen atoms on C-2), and glyceraldehyde, also behave as

TABLE 4.9

Donor and Acceptor Substrates for Transketolase[a]

Donors	Acceptors	Reference
D-Xylulose 5-P	D-Ribose 5-P	Racker (1961a)
D-Fructose 6-P	D-Deoxyribose 5-P	
D-Sedoheptulose 7-P	D-Glyceraldehyde 3-P	
D-Xylulose	D-Erythrose 4-P	
L-Erythrulose	Formaldehyde	
Octulose 8-P	Glycolaldehyde	
	Glyceraldehyde	
Hydroxypyruvate	D-Ribose	Villafranca and Axelrod (1971)
D-Fructose	L-Lyxose	
L-Sorbose	D-Xylose	
D-Sedoheptulose	L-Arabinose	
	D-Glucose 6-P	Horecker and Smyrniotis (1953a)
	D-Erythrose 4-homophosphonate	Le Marechal *et al.* (1980)
	4-Chloronitrosobenzene	Baden *et al.* (1980)
Dihydroxyacetone		Usmanov and Kochetov (1982, 1983a)

[a] Note, where no configuration is given for a compound existing in D and L forms, the isomer used was not specified by the author.

acceptor substrates. The glyceraldehyde reported to serve as a substrate was probably a racemic mixture of the D and L forms (Racker *et al.*, 1953), and it seems likely that only the D form was active. In this respect, L-glyceraldehyde 3-phosphate was inactive (Datta and Racker, 1961a).

It was suggested by Williams *et al.*, (1978a) that D-arabinose 5-phosphate can be utilized as an acceptor substrate for liver transketolase, and support for this was found in the results of Datta and Racker (1961a). However, D-arabinose 5-phosphate has the L configuration at C-2 and does not fit into the general pattern of acceptor substrate structures outlined previously, and D-arabinose itself has been shown not to be an acceptor (Villafranca and Axelrod, 1971). Further experiments have confirmed that D-arabinose 5-phosphate was not a substrate for the enzyme from *Candida utilis* (Wood and Gascon, 1980; Wood, 1981a) or rat liver (Wood and Gascon, 1980; Paoletti, 1983).

There is a certain variation in the ability of the enzyme from various sources to utilize the different substrates listed in Table 4.9. Thus, hydroxypyruvate serves as a donor substrate for the yeast (Dickens and Williamson, 1958; Datta and Racker, 1961a) and spinach (Villafranca and Axelrod, 1971) enzymes, but not for the rat liver enzyme (Sable, 1966; Wood and

Gascon, 1980; Paoletti, 1983). Glucose 6-phosphate is a feeble acceptor for the enzyme from yeast (Datta and Racker, 1961a; Wood and Gascon, 1980), although this conclusion has been questioned (Bonsignore *et al.*, 1962), but it is not an acceptor for the enzyme from rat liver (Paoletti, 1983) or pig liver (Simpson, 1960). The pig liver enzyme was reported not to react with aldopentoses, hexoses, formaldehyde, glycolaldehyde, and DL-glyceraldehyde (Simpson, 1960).

Equilibrium constants for the reactions with a number of substrates have been reported by Simpson (1960) and by Racker (1961a).

Purification

Transketolase has been isolated in a state of high purity from yeast, spinach, erythrocytes, pig and rat liver, and wheat leaves (Table 4.10). Specific activities in the range 14–43 units/mg have been measured for the enzymes from various types of yeast. The enzymes from mammalian tissues and wheat have lower specific activities of the order of 1–2.5 units/mg, while those from erythrocytes and spinach (Villafranca and Axelrod, 1971; Takabe *et al.*, 1980) had values around 8–9 units/mg.

A unique type of transketolase was isolated from *Candida boidinii* grown on methanol. It catalyzed the formation of dihydroxyacetone and D-glyceraldehyde 3-phosphate from D-xylulose 5-phosphate and formaldehyde and acted on the higher aldehydes up to the seven-carbon compounds. Glycolaldehyde and glyceraldehyde were better acceptor substrates than ribose 5-phosphate, unlike the conventional type of transketolase. The latter was also present in cells grown on methanol and was the only enzyme in cells grown on glucose, xylose, and ethanol. The new transketolase was only present in cells grown on methanol and has been christened dihydroxyacetone synthase (Waites and Quayle, 1981, 1983; Kato *et al.*, 1982).

A number of workers have investigated the use of affinity adsorbents for the purification of transketolase. Thiamine pyrophosphate diazo-coupled to *ortho*-aminophenyl-Sepharose was introduced by Klein and Brand (1977), and Specht (1977) utilized a thiamine pyrophosphate-Sepharose used previously in the purification of pyruvate oxidase (O'Brien *et al.*, 1976). The increases in specific activity obtained by their use were not great, and it has been questioned whether binding of the enzyme occurred by virtue of an affinity for thiamine pyrophosphate or by ion exchange (Wood and Fletcher, 1978). Eight possible affinity adsorbents were prepared by Wood and Fletcher (1978), but only ribose 5-phosphate-Sepharose and neopyrithiamine-Sepharose had any affinity for the yeast enzymes. The former was later used in the purification of the enzyme from *Candida utilis* (Wood, 1981a).

TABLE 4.10

Molecular Weight and Subunit Structure of Transketolase from Various Sources

Source	Molecular weight	No. of subunits	Molecular weight of subunits	Reference
Baker's yeast	—	2	60,000 (+ 29,000)	Kochetov and Belyaeva (1972)
Baker's yeast	140,000 ± 1000	2	70,000	Heinrich and Wiss (1971b), Heinrich (1973)
Baker's yeast	158,000 ± 4000	2	75,000–78,000	Cavalieri *et al.* (1975)
Baker's yeast	140,000	—	—	Chernyak *et al.* (1977)
Baker's yeast	159,000 ± 6000	2	—	Belyaeva *et al.* (1978)
Brewer's yeast	100,000 ± 6000	2	50,000 ± 3000	Saitou *et al.* (1974)
Candida utilis	163,000 ± 4500	2	78,080	Klein and Brand (1977)
Candida utilis	140,000	2	69,000–71,000	Specht (1977)
Candida utilis	—	—	81,000	Wood (1981a)
Human erythrocytes	136,000 ± 7000	—	—	Heinrich and Wiss (1971b)
Human erythrocytes	—	—	70,000	Schellenberg *et al.* (1982)
Rat liver	130,000	2	70,000	Kochetov and Minin (1978)
Rat liver	—	2	70,000	Gorbach *et al.* (1981)
Rat liver	139,000	2	69,000	Paoletti (1983)
Pig liver	138,000 ± 3,000	4	52,000–56,000 27,000–29,000	Philippov *et al.* (1979, 1980a)
Spinach	100,000 ± 10,000	1	—	Villafranca and Axelrod (1971)
Spinach	150,000	4	37,500	Murphy and Walker (1982)
Wheat	150,000 ± 4,000	4	37,600	Murphy and Walker (1982)
Candida boidinii KD1[a]	145,000	2	76,000	Bystrykh *et al.* (1981)
Candida boidinii 2201 (Kloeckera sp.)[a]	190,000	4	55,000	Kato *et al.* (1982)
Candida boidinii CBS 5777[a]	107,500	2	65,000	Waites and Quayle (1983)
Candida boidinii CBS 5777[b]	135,000	2	68,000	Waites and Quayle (1983)

[a] Grown on a one-carbon substrate.

[b] Grown on glucose, xylose, or ethanol.

Stability

Crystal suspensions and lyophilized preparations of transketolase are stable for several months when stored at low temperatures. Specht (1977) has determined temperature-stability curves for the *C. utilis* enzyme and showed that the enzyme was stabilized by the presence of the cofactors, magnesium and thiamine pyrophosphate. The enzyme purified from this source was stable in solution with dithiothreitol and added cofactors for several weeks at 5°C, for several months at −15°C, and for over a year when lyophilized and stored at −15°C (Wood, 1981a). The Baker's yeast enzyme was stored in 0.25 *M* glycerol + 0.1 m*M* dithiothreitol at −20°C but lost 33% of its activity in 2 weeks (Cavalieri *et al.*, 1975). The pig liver enzyme could be stored for 3 months at −20°C at pH 7.8–8.0 with little loss of activity, and the enzyme was relatively stable at 40°C in this pH range. Some of the activity lost on storage could be restored by incubation with cysteine, and, although the coenzyme could not readily be split off, storage in the presence of thiamine pyrophosphate improved its stability (Simpson, 1960; Philippov *et al.*, 1980a). The enzyme from human erythrocytes was very stable, and it was not affected by freezing and thawing (Heinrich and Wiss, 1971a; Warnock and Prudhomme, 1982). Stability-pH curves have been published for the pig liver enzyme (Simpson, 1960) and for the enzyme from *C. utilis* (Klein and Brand, 1977). The latter enzyme was not inactivated by 0.1 m*M* phenylmethylsulfonyl fluoride in 18 hr (Wood, 1981a).

Assay Methods

Assay methods have been discussed by Racker (1961a). One of the most commonly used assays is shown in Scheme 11. A useful comparison of the assay of rat heart extracts and of pure yeast transketolase by five different methods was made by Williams's group. In rat heart extracts the assays were complicated by the presence of NADH oxidase and fructose bisphosphatase. Interference from the former was avoided by using erythrose 4-phosphate as an acceptor substrate and coupling fructose 6-phosphate formation to NADP reduction (Scheme 12). Unfortunately, the triose phosphate that was also produced was condensed by aldolase present in the extract to form

$$\begin{gathered} \text{Xu—5—P} + \text{R—5—P} \rightleftharpoons \text{S—7—P} + \text{GAP} \\ \text{GAP} \rightleftharpoons \text{DHAP} \\ \text{DHAP} + \text{NADH} \rightleftharpoons \text{GlP} + \text{NAD}^+ \end{gathered}$$

Scheme 11. Assay of transketolase using xylulose 5-phosphate, ribose 5-phosphate, triose-phosphate isomerase, NADH, and glycerophosphate dehydrogenase.

$$\text{Xu—5—P} + \text{Er—4—P} \rightleftharpoons \text{GAP} + \text{F—6—P}$$
$$\text{F—6—P} \rightleftharpoons \text{G—6—P}$$
$$\text{G—6—P} + \text{NADP}^+ \rightarrow \text{6—PG} + \text{NADPH} + \text{H}^+$$

Scheme 12. Assay of transketolase using xylulose 5-phosphate, erythrose 4-phosphate, glucose-6-phosphate isomerase, $NADP^+$, and glucose-6-phosphate dehydrogenase.

fructose bisphosphate, which was then split by fructose bisphosphatase to form additional fructose 6-phosphate.

The formation of triose phosphate could be avoided by using 3-hydroxypyruvate as a donor substrate so that the second product was CO_2 (Scheme 13). The rate obtained with pure transketolase was only one-fifth of the rate with xylulose 5-phosphate as the donor substrate, but it was believed to be a true measure of the transketolase present (Clark *et al.*, 1972a).

A number of new assays have been developed in Kochetov's laboratory. The first of these involves using hydroxypyruvate as a donor substrate and following its rate of disappearance at 220–240 nm (Kochetov and Philippov, 1972, 1973). The K_m for hydroxypyruvate is high, and rather large concentrations of this substrate are required to saturate the enzyme. The assay cannot be used with those types of transketolase that do not utilize hydroxypyruvate as a substrate.

A second assay follows the condensation of hydroxypyruvate with glycolaldehyde to form D-erythrulose. The open-chain form of the latter has a peak in its circular dichroism spectrum at 275 nm that enables its formation to be followed with suitable equipment (Kochetov *et al.*, 1978). The third new assay is more general in its application than the others and may be used with any of the donor substrates. The carbanion glycolaldehyde–transketolase intermediate is oxidized in the presence of hexacyanoferrate(III) to glycolate, and the rate of reduction of the iron reagent is followed at 420 nm. No auxiliary enzyme or acceptor substrate is required, but the transketolase is slowly inactivated during the reaction (Usmanov and Kochetov, 1981; Kochetov, 1982a).

Molecular Properties

Molecular Weight and Subunit Composition

The molecular weights and subunit composition of transketolase from a number of sources are given in Table 4.10. Molecular weights are all in the

$$\text{3-hydroxypyruvate} + \text{Er—4—P} \rightleftharpoons \text{CO}_2 + \text{F—6—P}$$
$$\text{F—6—P} \rightleftharpoons \text{G—6—P}$$
$$\text{G—6—P} + \text{NADP}^+ \rightarrow \text{6—PG} + \text{NADPH} + \text{H}^+$$

Scheme 13. Assay of transketolase using 3-hydroxypyruvate, erythrose 4-phosphate, glucose-6-phosphate isomerase, $NADP^+$, and glucose-6-phosphate dehydrogenase.

range 100,000–163,000, and the enzyme may consist of one, two, or four subunits. In general, subunits had a molecular weight of 50,000–81,000; however, the pig liver enzyme was reported to contain two types of subunits, a large one of molecular weight 52,000–56,000 and a smaller one of about half this size (Philippov *et al.*, 1979, 1980a). A fragment of molecular weight 29,000 has also been observed with the Baker's yeast enzyme (Kochetov and Belyaeva, 1972).

Subunit Interactions

Unless otherwise stated, all the results described in this section were obtained with the Baker's yeast enzyme.

Transketolase at concentrations below 120 μg/ml was found to dissociate into its subunits at 5 °C in the absence of thiamine pyrophosphate. When the coenzyme was present, dissociation did not occur, even at concentrations as low as 0.06 μg/ml. The dimer was reported to be the active species, and the monomer appeared to be inactive (Cavalieri *et al.*, 1975). These results were confirmed when reversible dissociation into two subunits was observed at 20 °C and pH 7.6 at concentrations below 200 μg/ml. Higher temperatures appeared to promote dissociation, and at pH values outside the range 5–10.5 the process became irreversible. At 5 °C and pH 7.6 and concentrations below 5 μg/ml, the apparent molecular weight determined by gel filtration dropped from 159,000 to 134,000, suggesting that some dissociation was occurring (Belyaeva *et al.*, 1978). Similar behavior was observed by Heinrich *et al.*, (1972), who attributed it to a hydrophilic reaction between transketolase and the Sephadex matrix. At a high pH of 9.0 and at concentrations below 90 μg/ml, a 2.8 S component (as opposed to 7.6 S for the 140,000 dalton dimer) appeared, indicating that dissociation could occur under these conditions (Kochetov and Belyaeva, 1972).

Kochetov and Soloveva (1977) coupled transketolase to cyanogen-bromide–activated Sepharose and showed that the stability of the enzyme was considerably enhanced and the pH-activity curve and K_m values of the pentose phosphate substrates remained essentially unchanged. Subsequently, it was shown that the enzyme could be bound by one subunit and that in the bound form the subunit had the same specific activity as the free enzyme. Bound subunits could interact with subunits of transketolase in solution to form the complete holoenzyme (Kochetov and Soloveva, 1978; Soloveva and Kochetov, 1979). Reduced sulfhydryl groups appeared to be essential for reassociation, and treatment with *N*-ethylmaleimide destroyed the ability of bound subunits to interact. Reassociation was most rapid in the range pH 7.9–9.2, and calcium and thiamine pyrophosphate, or calcium alone, promoted the recombination (Soloveva and Kochetov, 1980). Kinetic studies of the reconstitution of holotransketolase led to the conclusion that

in solution the monomer was either inactive, or less active, than the dimeric form of the enzyme (Egan and Sable, 1981).

The spinach enzyme was reported to have a molecular weight of 100,000 and to consist of one subunit (Villafranca and Axelrod, 1971). However, Murphy and Walker (1982) disputed this and reported that spinach transketolase had a molecular weight of 150,000 and was a tetramer that readily dissociated into dimers of molecular weight 75,000. They found the wheat enzyme to be very similar in molecular weight and subunit composition.

In experiments with the rat liver enzyme it was shown that the holoenzyme could only be dissociated into its subunits by treatment with acid at pH 4.0. The subunits, when attached to Sepharose, could not bind thiamine pyrophosphate but were catalytically active only when it was present in solution. In the presence of thiamine pyrophosphate and calcium, the bound liver subunits were able to form active dimers with subunits of the Baker's yeast enzyme (Minin and Kochetov, 1981).

Amino Acid Composition

The amino acid composition has been determined for the enzymes from Baker's yeast (Kochetov *et al.*, 1973a, Kochetov, 1982b), *Candida utilis* (Specht, 1977), and pig liver (Kochetov, 1982*b*). Arginine has been identified as the *N*-terminal amino acid of the enzyme from *C. utilis* (Specht, 1977) and from Baker's yeast (Heinrich and Wiss, 1971b), and two arginine residues were reported to be essential for catalytic activity (Kremer *et al.*, 1980; Kochetov, 1982c).

Tertiary Structure

From measurements of optical rotatory dispersion the enzyme protein was reported to contain 30–31% helical structure (Kochetov and Usmanov, 1970b), while calculations from CD measurements led to values of 20% α-helix, 40% β-sheet, and 40% random coil (Kochetov *et al.*, 1971b). Acid and alkali denaturation could be reversed by incubation in tris buffer at pH 8 (Kochetov and Usmanov, 1970b). A preliminary X-ray study of the Baker's yeast enzyme has been made (Konareva *et al.*, 1983).

Complexes of Transketolase with Other Enzymes

Complex formation between transketolase and transaldolase of *Candida utilis* was reported by Kiely *et al.*, (1969), while complexes between transketolase and glyceraldehyde phosphate dehydrogenase of Baker's yeast were noted during cation-exchange chromatography (Kochetov *et al.*, 1970a; Kochetov and Chernov, 1970, 1971), and a transketolase–glyceraldehyde phosphate dehydrogenase complex was identified as a separate band after polyacrylamide gel electrophoresis (Kochetov *et al.*, 1970a). The latter com-

plex was isolated from Baker's yeast, and its dissociation was studied (Saitou *et al.*, 1974). A similar complex that could only be dissociated with difficulty has been purified from human erythrocytes (Heinrich and Wiss, 1971a).

Klein and Brand (1977) found no evidence of complex formation during the purification of transketolase from *Candida utilis* and suggested that the multiple peaks reported by Kiely *et al.*, (1969) were elution artifacts. This was refuted by Wood (1981a), who pointed out that multiple peaks had consistently been observed with impure preparations of the *Candida utilis* enzyme after gel filtration, chromatography on ion-exchangers, and affinity adsorbents (Wood and Fletcher, 1978; Wood, 1981a), and multiple bands had been observed on electrophoresis gels stained for transketolase activity (Wood and Muzariri, 1981).

Although transketolase from *Candida utilis* may be isolated in multimolecular forms associated with other enzymes, as it is purified this multiplicity disappears, and the pure enzyme gives a single peak when chromatographed on DEAE-Sephadex and a single band on electrophoresis (Wood, 1981a). If we consider the possibility that the formation of glyceraldehyde 3-phosphate by transketolase and its removal by glyceraldehyde-3-phosphate dehydrogenase are coupled together and remembering that transketolase and transaldolase can operate as a coupled system (see Chapter 2), then it is plausible that the formation of complexes between these three enzymes may have a functional significance (Wood et al., 1985).

Electrophoresis and Isoelectric Point

Isoelectric points have been reported as follows: Baker's yeast enzyme, 6.5–7.0 (Philippov *et al.*, 1980a; Kochetov and Chernov, 1971); enzyme from *Candida utilis*, 4.7–5.0 (Specht, 1977), 4.8 (Klein and Brand, 1977); pig liver enzyme, 7.6–7.8 (Philippov *et al.*, 1980a; Kochetov, 1982b); enzyme from human erythrocytes, 8.5 (Heinrich and Wiss, 1971a).

Three methods for the detection of transketolase activity after electrophoresis in a polyacrylamide gel have been published (Kochetov *et al.*, 1970a; Anderson *et al.*, 1975; Wood and Muzariri, 1981). The first method was used to stain free transketolase and a transketolase–glyceraldehyde-3-phosphate dehydrogenase complex, but using this stain Wood and Muzariri (1981) were unable to detect pure transketolase after electrophoresis, and they introduced an improved procedure that used a starch indicator–gel-containing substrate and the coupling enzyme. The first method has been used successfully for the staining of transketolase isozymes in an agarose gel (Kaczmarek and Nixon, 1983). No results with the second procedure appear to have been described.

Multimolecular Forms

Kaczmarek and Nixon (1983) have identified eight different species of transketolase in human erythrocytes by isoelectric focusing on agar gel. The species had isoelectric points of 6.6, 7.3, 7.5, 7.8, 8.1, 8.2, 8.4, and 9.2, and 6 different combinations of these were present among the 25 subjects tested. Differences between these species in their affinity for TPP were noted, so a reassessment of the relationship between nutritional status and the "TPP effect" might be required (see Chapter 10).

In pea and rye leaves, 90% of the transketolase appeared to be present in the chloroplasts and 10% or less in the cytoplasm. Contrary to expectations, no multiple forms of the enzyme could be detected in leaf extracts of rye, wheat, pea, and spinach (Feierabend and Gringel, 1983).

Catalytic Properties

Cofactors

As far as is known, transketolase from whatever source requires thiamine pyrophosphate as a cofactor. The coenzyme may be either strongly bound and incapable of being removed by dialysis or bound in a form that can be dialyzed off. In addition, the enzyme may require metal ions for its activity. Thiamine pyrophosphate may be replaced as the coenzyme by 2'-ethylthiamine pyrophosphate (Heinrich *et al.*, 1972).

Baker's-yeast transketolase is able to bind from two to nine molecules of thiamine pyrophosphate per molecule of enzyme (Datta and Racker, 1961b); however, only two molecules seem to be required for full enzymic activity and are present in the purified holoenzyme (Kochetov *et al.*, 1972; Heinrich, 1973; Kochetov *et al.*, 1976). Similarly, the enzymes from human erythrocytes (Heinrich and Wiss, 1971a), rat liver (Kochetov and Minin, 1978), and pig liver (Philippov *et al.*, 1979, 1980a) all contained two firmly bound coenzyme molecules per molecule.

Metal Ion Requirement

The ability of various divalent ions to restore the activity of Baker's yeast transketolase that had lost 80% of its original activity by storage was tested and found to be effective in the following order: $Mn^{2+} > Mg^{2+} > Fe^{2+} = Zn^{2+} = Ni^{2+} > Co^{2+} = Ca^{2+} > Cd^{2+} > Hg^{2+} > Cu^{2+}$ (Kochetov *et al.*, 1969). Despite the relatively low position of calcium in this list, calcium was identified as the metal cofactor present in the enzyme (Kochetov and Philippov, 1970a, 1970b, 1970c). It was reported that EDTA inhibited the reconstitution of holotransketolase, apparently by complexing with the metal.

The resulting inhibition could be reversed by the addition of divalent ions, and the efficiency of activation was in the order $Mn^{2+} > Ca^{2+} > Mg^{2+}$ at concentrations below 3 m*M*, and in the order $Ca^{2+} > Mn^{2+} > Mg^{2+}$ at 3 m*M* (Saitou *et al.*, 1974). A similar order, $Ca^{2+} > Mn^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+}$ was observed by Heinrich *et al.*, (1972) at 5 m*M* concentrations.

The enzyme from *Candida utilis* requires magnesium ion as a cofactor (Klein and Brand, 1977), but the enzyme prepared from erythrocytes had no requirement for a divalent ion, although calcium and magnesium ions had a slight activating effect (Heinrich and Wiss, 1971a; Warnock and Prudhomme, 1982). Similarly, the rat and pig liver enzymes had no such requirement (Philippov, *et al.*, 1980a; Kochetov, 1982c; Paoletti, 1983), and only insignificant amounts of metal were present in the pig liver preparation (Philippov *et al.*, 1980a). The cytosolic wheat enzyme was insensitive to magnesium, but the activity of the chloroplast form varied with the concentration of magnesium ion (Murphy and Walker, 1982). Magnesium also appeared to be necessary for the activity of the spinach enzyme (Horecker and Smyrniotis, 1953a). Where a metal ion is required for activity, it seems to participate in the binding of thiamine pyrophosphate to the enzyme (Kochetov, 1982c).

Removal of Thiamine Pyrophosphate

Thiamine pyrophosphate could not be removed from spinach transketolase by simple dialysis but was split off by precipitation of the protein at 45% saturation by ammonium sulfate at pH 3 (Horecker and Smyrniotis, 1953a). It could only be removed from the Baker's yeast enzyme by exhaustive dialysis against phosphate buffer containing 0.6% EDTA at pH 7.4 (Racker *et al.*, 1953) or against 1.6 *M* ammonium sulfate at pH 7.8 (Datta and Racker, 1961a). The coenzyme of transketolase from *Candida utilis* was readily removed from dilute solutions by simple dialysis (Kiely *et al.*, 1969), but dialysis against EDTA was necessary for its complete removal from concentrated solutions (Wood and Fletcher, 1978). Klein and Brand (1977) reported that the coenzyme was almost completely removed during their purification procedure, and Specht (1977) found dialysis or gel filtration sufficient to remove it completely.

The conditions necessary for removal of thiamine pyrophosphate from the Baker's yeast enzyme were investigated by Kochetov and Izotova (1970), who found dialysis against ammonium sulfate or ammonium chloride at pH values above 8.5 most effective by causing displacement of the coenzyme by ammonium ion.

Thiamine pyrophosphate could not easily be removed from the transketolases of mammalian tissues without destroying the enzyme, and it appears to be held very strongly, possibly by a covalent bond. Heinrich and Wiss

(1971a) were unable to remove it from the erythrocyte enzyme by dialysis against EDTA–KCl or by gel filtration, but it could be split off by treatment with ammonium sulfate at pH 3.5 (Warnock and Prudhomme, 1982).

It could be split from the rat liver enzyme by boiling (Kochetov and Minin, 1978) or by treatment with 1.7 *M* perchloric acid (Philippov 1980b). Not even boiling with *N* hydrochloric acid would remove it from the pig liver enzyme (Philippov *et al.*, 1980a), although addition of the coenzyme would stabilize the enzyme (Simpson, 1960; Philippov *et al.*, 1980a). Fragments believed to contain thiamine pyrophosphate bound to peptides were obtained after enzymic digestion of the pig liver enzyme (Philippov *et al.*, 1980b). However, it was later shown that the coenzyme was *not* held by a covalent bond and could be removed by boiling for 2 min or treatment with 10% trichloracetic acid (Voskoboev and Gritsenko, 1981). It could also be split off by standing at 0°C for 60 min in the pH range 4.5–6.0. The holoenzyme could be reconstituted at pH 6.0–6.4, but reconstitution was only complete at pH values of 7.6 and above (Tomita *et al.*, 1979). The coenzyme could only be removed from mouse brain transketolase by treatment, first with an alkaline phosphate buffer and then with ammonium sulfate, pH 3.5, and it was suggested that there were two ionic binding sites for the coenzyme, one with an acid pK, the other with an alkaline pK (Blass *et al.*, 1982).

Binding of Thiamine Pyrophosphate and Analogs

Many investigators have studied the binding of cofactors and substrates and the groups on the enzyme responsible for this binding. Unless stated otherwise these investigations have been carried out with the Baker's yeast enzyme.

Binding of thiamine pyrophosphate or certain analogs to the apoenzyme produces changes in the UV spectrum at 320 nm (Kochetov and Usmanov, 1970a; Kochetov *et al.*, 1976) in the ORD and CD spectra (Kochetov *et al.*, 1970b, 1970c, 1971b; Heinrich *et al.*, 1971, 1974) and in the proton magnetic resonance spectrum (Heinrich *et al.*, 1974). Binding to the enzyme also changes the fluorescence of the protein at 325–350 nm (Heinrich *et al.*, 1972; Kochetov *et al.*, 1975a). All of these phenomena, together with activity measurements, have been used to follow binding to the protein and, in some cases, to measure binding constants (Tables 4.11 and 4.12).

Silver ion was found to react with the thiol groups of transketolase and could be displaced by thiamine pyrophosphate (Kochetov and Lutavinova, 1966), and two cysteine residues per subunit were identified as being necessary for the binding of thiamine pyrophosphate to the *Candida utilis* enzyme (Specht, 1977). Transketolase is inhibited by some reagents reacting with thiol groups (Table 4.14), and free thiol groups appear to be necessary for

TABLE 4.11

Michaelis, Inhibition, and Binding Constants for Thiamine Pyrophosphate and Its Analogs with the Baker's Yeast Enzyme

Compound	K_i (μM)	K^a (μM)
Thiamine pyrophosphate	—	1.00[b](M)
Thiamine pyrophosphate	—	1.06[b](F)
Thiamine pyrophosphate	—	0.28[c](M)
Thiamine thiazolone pyrophosphate	0.028[d]	
Oxythiamine pyrophosphate	0.030[b]	0.035[b](F)
Tetrahydrothiamine pyrophosphate	0.40[b]	0.45[b](F)
Thiazole pyrophosphate	5.4[c]	—
2′-Ethylthiamine pyrophosphate	—	5.5[b](M)
Thiochrome pyrophosphate	6.3[b]	
Pyrithiamine pyrophosphate	110[b]	
Pyrophosphate ion	4200[b]	
Pyrophosphate ion	280[c]	
Thiamine monophosphate	2000[c]	
Thiazole	5000[c]	
Thiamine	35000[c]	
Ammonium ion	42000[c]	

[a] F, binding constant determined from fluorescence quenching; M, Michaelis constant.
[b] Heinrich *et al.* (1972).
[c] Kochetov *et al.* (1971a); Kochetov and Izotova (1973b)
[d] Shreve *et al.* (1983).

the binding of coenzyme to newly synthesized apoenzyme (Böhm *et al.*, 1973).

Similar changes to those observed with the apoenzyme were observed in the UV and CD spectra when thiamine pyrophosphate was mixed with tryptophan (Heinrich *et al.*, 1971) or when thiamine pyrophosphate or its analogs interacted with indoleacetic acid (Heinrich *et al.*, 1974), and it was concluded that the formation of a charge transfer complex between the coenzyme and a tryptophan residue in the enzyme protein were responsible (Kochetov and Usmanov, 1970a; Heinrich *et al.*, 1971, 1974). The tryptophan-specific reagent dimethyl-(2-hydroxy-5-nitrobenzyl)-sulphonium chloride produced a 90% loss of activity of the apoenzyme but no loss with the holoenzyme. The presence of magnesium and thiamine pyrophosphate also protected the enzyme against tryptic digestion (Heinrich *et al.*, 1973).

Among the various analogs tested for their ability to bind to indoleacetate, only thiamine pyrophosphate formed a charge-transfer complex. It was concluded that the formation of such a complex might be of importance for catalysis of the reaction but was not essential for coenzyme binding, since

TABLE 4.12

Michaelis and Dissociation Constants for the Thiamine Phosphate–Transketolase Complex

Type of constant	Value (μM)	Reference
Baker's yeast enzyme		
K_m (0.1 mM Ca^{2+})	33	Kochetov and Philippov (1970c)
K_m (5 mM Ca^{2+})	4	
K_m (no Mg^{2+})	30	
K_m (70 μM Mg^{2+})	20	
K_m (14 mM Ca^{2+})	0.3	Kochetov and Izotova (1973b)
K_m	0.6	Kochetov *et al.* (1971a)
K_m (no Mg^{2+})	4.8	Heinrich *et al.* (1972)
K_m (4 mM Mg^{2+})	1.0	
Dissociation (5 mM Mg^{2+})	1.1	
Dissociation	1.4	Heinrich and Schmidt (1973)
Dissociation (1 mM Ca^{2+})	0.032, 0.25	Kochetov *et al.* (1975b)
Dissociation (1 mM Mg^{2+})	0.03	
K_m (3 mM Ca^{2+})	0.08, 0.5	Egan and Sable (1981)
K_m (3 mM Mg^{2+})	0.4, 5.7	
Others[a]		
K_m (3.7 mM Mg^{++}, C)	5.4	Klein and Brand (1977)
K_m (1 mM Mg^{++}, M)	7	Blass *et al.* (1982)
K_m (2 mM Mg^{++}, E)	0.4	Warnock and Prudhomme (1982)

[a] C, *Candida utilis;* M, mouse brain; E, human erythrocytes.

analogs such as oxythiamine or tetrahydrothiamine pyrophosphates bound more tightly to the enzyme than thiamine pyrophosphate but did not produce the characteristic charge-transfer band in the CD spectrum. Furthermore, tetrahydrothiamine appeared incapable of forming a charge-transfer complex with the indole ring, whereas pyrithiamine formed the strongest charge-transfer complex of the derivatives studied but was only weakly bound to apotransketolase (Heinrich *et al.*, 1974).

Studies of the binding to apotransketolase of the pyrophosphates of thiamine, 3-methylthiamine, 3-benzylthiamine, and thiazole led to the similar conclusion that the positively charged nitrogen of the thiazole ring played little part in coenzyme binding but was essential for catalytic activity (Tomita *et al.*, 1974).

Heinrich *et al.* (1972) measured binding constants (by fluorescence quenching) and inhibition constants (from activity measurements) for thia-

mine pyrophosphate and a number of analogs. The values obtained are listed in Tables 4.11 and 4.12. From the results it was concluded that hydrophobic interactions were not important in the coenzyme binding and that there must be a pyrophosphate binding site on the enzyme, a conclusion reached earlier by Kochetov *et al.*, (1971a). Sulfate ion was found to activate the enzyme at concentrations below 20 m*M* and inhibit at higher concentrations. It lowered the binding constant for thiamine pyrophosphate, probably by competition for the pyrophosphate binding site.

Binding of a donor substrate to the enzyme abolished the ellipticity band at 325 nm in the CD spectrum, whereas acceptor substrates had no effect (Heinrich *et al.*, 1971). A similar change was observed in the absorbance spectrum (Kochetov *et al.*, 1973b) and in the number of accessible tyrosine and tryptophan residues (Usmanov and Kochetov, 1978).

Histidine residues were identified as being present at the active site (Kochetov and Lutavinova, 1966), and photoinactivation studies indicated that they were involved in the binding of thiamine pyrophosphate (Kochetov and Kobylyanskaya, 1970). Diethylpyrocarbonate reacted specifically with histidine residues of apotransketolase from *Candida utilis* (Specht, 1977) and Baker's yeast (Kochetov, 1982c) inactivating the enzymes, and the reaction was markedly inhibited by the presence of thiamine pyrophosphate. As pyrophosphate itself also protected the enzyme against inactivation, it was concluded that histidine was the pyrophosphate binding site (Kochetov, 1982c).

Saitou *et al.*, (1974) found that the reconstitution of the holoenzyme was inhibited by phenylglyoxal and suggested that an arginine residue at the active site was blocked by the reagent, thus preventing the binding of the coenzyme pyrophosphate group; however, Kremer *et al.*, (1980) were unable to confirm this. Their results, using phenylglyoxal and 2,3-butanedione to modify arginine groups showed that there was a single arginine residue per active site essential for activity. Modification of this arginine residue prevented the formation of the charge-transfer complex but may not necessarily have prevented the binding of thiamine pyrophosphate to the modified enzyme. The presence of either a donor or an acceptor substrate protected the enzyme against inactivation by 2,3-butanedione only when thiamine pyrophosphate was bound at the active site, and it was suggested that arginine residues are part of the active site and may be involved in the binding of substrates (Kochetov, 1982c). Later, it was shown that the arginine residues do not participate in substrate binding but are necessary for catalysis (Usmanov and Kochetov, 1983b).

The conditions for reconstitution of the holoenzyme have been investigated by several groups of Russian workers and others. When apotranske-

tolase was incubated with coenzyme and magnesium chloride the activity obtained was proportional to the amount of thiamine pyrophosphate up to 2 moles/mole enzyme (Kochetov *et al.*, 1972; Kochetov and Izotova, 1973a). Similar results were obtained by following protein fluorescence (Kochetov *et al.*, 1975a) and absorbance at 320 nm (Kochetov *et al.*, 1976). It was also shown that there were two major binding sites on the enzyme, one binding the coenzyme much more strongly than the other. In the presence of magnesium a positive cooperativity was found. The major part of the activity appeared when only one molecule of coenzyme was bound per molecule of enzyme. The decrease in activity of the enzyme on storage appeared to be due to inactivation of the weaker binding site (Kochetov *et al.*, 1975b; Meshalkina and Kochetov, 1979). From spectroscopic experiments in mixed solvents it was concluded that the binding of coenzyme was accompanied by a drop in the number of accessible tyrosine and tryptophan residues by half. When a donor substrate was added the number of accessible residues was restored. It was concluded that at the stage of glycolaldehyde transfer to the acceptor substrate, the active site was in an aqueous phase (Usmanov and Kochetov, 1978).

Reconstitution of the holoenzyme was also investigated by Heinrich *et al.*, (1972), who found, as reported by Kochetov and Philippov (1970c) and Kochetov (1982c), that thiamine pyrophosphate, in the absence of metal ions, could restore a considerable fraction of the total potential activity. At pH values above 6.5 the binding of cofactors was reversible; below this value they could not be removed by gel filtration. The K_m value for thiamine pyrophosphate became smaller at lower pH values and increased markedly at pH 8.0. In the temperature range 10–37°C, the K_m value showed a minimum at 28°C.

Kochetov (1982c) has interpreted the various binding experiments carried out by his group as indicating that one molecule of thiamine pyrophosphate binds to one active site that already contains one atom of firmly bound metal to give an activity of half the maximum. Binding of a second molecule of coenzyme to form the fully active holoenzyme requires the presence of free divalent cations. In the presence of inadequate amounts of the latter, binding of the coenzyme to the second active site results in less than maximal activity.

The lag time between the addition of apotransketolase to the reaction mixture and the attainment of a steady-state velocity has been studied by Egan and Sable (1981). They concluded that the binding of the coenzyme in the presence of magnesium or calcium ion was a process with negative cooperativity. In addition, a slow transition between two forms of the enzyme also appeared to contribute to the observed lag. Preincubation for 20

min with magnesium, thiamine pyrophosphate, and D-xylulose 5-phosphate resulted in abolition of the lag when the reaction was initiated by the addition of ribose 5-phosphate.

The binding of thiamine thiazolone pyrophosphate has been studied, and it was concluded that although it behaved as an effective transition state analog with pyruvate dehydrogenase, this was not the case with Baker's yeast transketolase, and the active site of the latter was more hydrophilic than that of the dehydrogenase (Shreve *et al.*, 1983).

Binding of Substrates

Very little has been learned of the binding of substrates to transketolase. As described previously, binding of donor substrates such as fructose 6-phosphate and hydroxypyruvate affected the charge transfer band around 325 nm (Heinrich *et al.*, 1971; Kochetov *et al.*, 1973b) and increased the number of accessible tyrosine and tryptophan residues (Usmanov and Kochetov, 1978), suggesting that the binding was accompanied by a pronounced change in conformation. The strong affinity of the acceptor site for D-ribose 5-phosphate is illustrated by the use of D-ribose 5-phosphate in an affinity adsorbent and of D-ribose 5-phosphate to elute the adsorbed enzyme (Wood and Fletcher, 1978; Wood, 1981a). The binding of substrates to the Baker's yeast enzyme resulted in changes in the CD spectrum that could be used to measure the degree of binding. Dihydroxyacetone was shown to be a donor substrate for the enzyme. An ionic group on the donor substrate was necessary for tight binding to the holoenzyme (Usmanov and Kochetov, 1982, 1983a).

Kinetic Measurements

The enzymes from Baker's yeast (Cavalieri, 1973), *Candida utilis* (Klein and Brand, 1977), *Candida boidinii* (Kato *et al.*, 1982) were all reported to follow a ping-pong mechanism.

Activity-pH curves have been published for the enzymes from Baker's yeast (Datta and Racker, 1961a), *Candida utilis* (Klein and Brand, 1977; Specht, 1977), pig liver (Simpson, 1960), rat liver (Paoletti, 1983), and human erythrocytes (Massod *et al.*, 1971; Warnock and Prudhomme, 1982). The values of the optimal pH are all between 6.8 and 7.9. In addition, pH optima have been reported of 8.0 for the pig liver enzyme (Philippov *et al.*, 1979, 1980a) and 7.0 for the enzyme from *Candida boidinii* (Kato *et al.*, 1982). The cytosolic wheat enzyme had an optimal pH of 8.5–9.0 and the chloroplast enzyme of pH 7.5–8.5 (Murphy and Walker, 1982).

Michaelis constants for substrates are given in Table 4.13 and for the coenzymes thiamine pyrophosphate and 2'-ethylthiamine pyrophosphate in Tables 4.11 and 4.12. Very few inhibition constants have been deter-

TABLE 4.13

Michaelis Constants for Substrates of Transketolase

Substrate	K_m (μM)	Source of enzyme	Reference
D-Xu-5-P	22	Rat liver	Gorbach *et al.* (1981)
	25	Rat liver	Paoletti (1983)
	68	*Candida utilis*	Kiely *et al.* (1969)
	77	*Candida utilis*	Wood (1981a)
	70	*Candida utilis*	Specht (1977)
	210	Baker's yeast	Datta and Racker (1961a)
	160	Rat liver	Novello and McLean (1968)
	130	Mouse brain	Blass *et al.* (1982)
	18	Rat brain	Kauffman (1972)
	230	*Entamoeba histolytica*	Susskind *et al.* (1982)
	180	Human erythrocytes	Warnock and Prudhomme (1982)
	1000[a]	*Candida boidinii*	Kato *et al.* (1982)
D-R-5-P	27	*Entamoeba histolytica*	Susskind *et al.* (1982)
	66	Rat liver	Paoletti (1983)
	300	Human erythrocytes	Warnock and Prudhomme (1982)
	300	*Candida utilis*	Specht (1977)
	330	Mouse brain	Blass *et al.* (1982)
	400	Baker's yeast	Datta and Racker (1961a)
	400	Spinach	Villafranca and Axelrod (1971)
	430	*Candida utilis*	Wood (1981a)
	2000[a]	*Candida boidinii*	Kato *et al.* (1982)
	4000[a]	*Candida boidinii*	
D-F-6-P	1800	Baker's yeast	Datta and Racker (1961a)
	3200	Spinach	Pontremoli *et al.* (1960)
D-Er-4-P	5	*Entamoeba histolytica*	Susskind *et al.* (1982)
	44	Rat liver	Novello and McLean (1968)
	50	*Candida utilis*	Specht (1977)
D-GAP	70	*Candida utilis*	Wood (1973b)
	4900	Baker's yeast	Datta and Racker (1961a)
	420[a]	*Candida boidinii*	Kato *et al.* (1982)
	1600	*Entamoeba histolytica*	Susskind *et al.* (1982)
Hydroxypyruvate	33000	Baker's yeast	Kochetov and Philippov (1973)
	250	Baker's yeast	Usmanov and Kochetov (1983a)
	42000	*Candida utilis*	Wood (1973b)
Other substrates			
Erythrulose	4.9	Baker's yeast	Datta and Racker (1961a)
D-Ribose	45	Spinach	Villafranca and Axelrod (1971)

(continued)

TABLE 4.13 *(Continued)*

Substrate	K_m (μM)	Source of enzyme	Reference
L-Lyxose	55	Spinach	Villafranca and Axelrod (1971)
L-Arabinose	120	Spinach	Villafranca and Axelrod (1971)
D-Xylose	230	Spinach	Villafranca and Axelrod (1971)
4-Chloronitroso-benzene	0.92	Baker's yeast	Baden *et al.* (1980)
Formaldehyde	0.43[a]	*Candida boidinii*	Kato *et al.* (1982)
Dihydroxyacetone	0.52[a]	*Candida boidinii*	
Dihydroxyacetone	28	Baker's yeast	Usmanov and Kochetov (1983a)
Fructose	15	Baker's yeast	Usmanov and Kochetov (1983a)

[a] Dihydroxyacetone synthetase.

mined, but a list of various reversible and irreversible inhibitors is given in Table 4.14. Values of the inhibition constants for the Baker's yeast enzyme as measured were 0.28 mM for pyrophosphate and 9.9 mM for orthophosphate, both competitive with the coenzyme (Kochetov and Izotova, 1973b). For transketolase from *Candida utilis*, K_i values for competitive inhibition of 4.9, 5.5, and 2.7 mM were obtained for orthophosphate, sulfate, and arsenate, respectively, but the variable substrate was not specified (Klein and Brand, 1977). Wood (1981a) found mixed inhibition of this enzyme by D-arabinose 5-phosphate ($K_i = 1.4$ mM, $K_i' = 2.6$ mM, variable substrate ribose 5-phosphate; $K_i = 0.82$ mM, $K_i' = 3.6$ mM, variable substrate xylulose 5-phosphate) and by D-glucose 6-phosphate ($K_i = 9.8$ mM, $K_i' = 17$ mM, variable substrate ribose 5-phosphate; $K_i = 3.6$ mM, $K_i' = 13$ mM, variable substrate xylulose 5-phosphate). Oxythiamine ($K_i = 1.4$ mM) and pyrithiamine ($K_i = 4.3$ mM) were inhibitors competitive with the coenzyme (Wood and Fletcher, 1978).

Activation of the Baker's yeast enzyme by sulfate ions was reported by Heinrich *et al.*, (1972), but no such effect was apparent with the enzyme from *Candida utilis* (Klein and Brand, 1977).

Reaction Mechanism

The mechanism of the transketolase reaction has been described in detail by Horecker (1964).

TABLE 4.14

Substances Reported to Inhibit Transketolase Activity[a]

Compound	Enzyme source	Reference
	A. Reversible inhibitors	
Phosphate	Baker's yeast	Datta and Racker (1961a), Kochetov and Izotova (1973b)
Phosphate	*Candida utilis*	Klein and Brand (1977), Wood (1981a)
Phosphate	Spinach	Pontremoli *et al.* (1960)
Phosphate	Human erythrocytes	Heinrich and Wiss (1971a), Dische and Igals (1963)
Phosphate	Rat liver	Gorbach *et al.* (1981)
Sulfate	Baker's yeast	Datta and Racker (1961a), Kremer *et al.* (1980)
Sulfate	*Candida utilis*	Klein and Brand (1977), Wood (1981a)
Sulfate	Human erythrocytes	Heinrich and Wiss (1971a), Dische and Igals (1963)
Sulfate	Rat liver	Gorbach *et al.* (1981)
Arsenate	*Candida utilis*	Klein and Brand (1977), Wood (1981a)
Arsenate	Rat liver	Gorbach *et al.* (1981)
D-Er-4-P	Human erythrocytes	Dische and Igals (1961)
D-Er-4-homo-P	Baker's yeast	LeMarechal *et al.* (1980)
D-Ara-5-P	*Candida utilis*	Wood and Gascon (1980), Wood (1981a)
D-G-6-P	*Candida utilis*	Wood and Gascon (1980), Wood (1981a)
D-xylose	Rat erythrocytes	Bössler and Stechert (1965)
2,3-PPGA	Human erythrocytes	Dische and Igals (1963)
Hydrazine	Baker's yeast	Simpson and Bhuyan (1962)
Oxythiamine	*Candida utilis*	Wood and Fletcher (1978)
Neopyrithiamine	*Candida utilis*	Wood and Fletcher (1978)
Urea	Baker's yeast	Heinrich and Wiss (1971b)
Mercaptoethanol	Baker's yeast	Heinrich and Wiss (1971b)
Mercaptoethanol	Human erythrocytes	Heinrich and Wiss (1971a)
Phenyl phosphate	Baker's yeast	Kremer *et al.* (1980)
	B. Irreversible inhibitors	
p-CMB	*Candida utilis*	Specht (1977), Wood (1981a)
DTNB	*Candida utilis*	Specht (1977)
Iodoacetamide	Pig liver	Simpson (1960)
N-Ethylmaleimide	Pig liver	Simpson (1960)
Hg^{2+}, Cu^{2+}, Pb^{2+}	Pig liver	Simpson (1960)
p-CMB	*Candida boidinii*	Kato *et al.* (1982)
Idoacetate	*Candida boidinii*	Kato *et al.* (1982)
Hg^{2+}	*Candida boidinii*	Kato *et al.* (1982)

[a] See also Table 4.11.

Spinach chloroplasts are able to form glycolate from fructose 6-phosphate, and it was shown that purified transketolase could catalyze a similar reaction in the presence of a suitable oxidant such as ferricyanide or ferredoxin and NADP (Bradbeer and Racker, 1961; Shain and Gibbs, 1971). The production of glycolate appeared to result from the oxidation of the carbanion intermediate of the transketolase reaction. Glycolate and erythrose 4-phosphate were produced from fructose 6-phosphate, and glycolate and glyceraldehyde 3-phosphate were formed when xylulose 5-phosphate was the substrate. The rate of the reaction was only 0.2–0.3% of the normal transfer reaction from fructose 6-phosphate to erythrose 4-phosphate (Christen and Gasser, 1980; Takabe *et al.*, 1980), and at the same time, the enzyme protein was slowly inactivated by subsequent reaction of the oxidized enzyme–substrate complex with amino acid side chains at the active site (Christen *et al.*, 1976; Christen, 1977). The oxidizing species was suggested to be hydrogen peroxide (Shain and Gibbs, 1971), but it was more probably superoxide, as the reaction was strongly inhibited by superoxide dismutase. It was consequently suggested that transketolase might function in addition to the dismutase as a secondary scavenger of superoxide in chloroplast stroma (Asami and Akazawa, 1977; Takabe *et al.*, 1980). From their studies, Krause *et al.*, (1977) concluded that transketolase was not involved in the formation of glycolate in intact plants. The reaction using ferricyanide as an oxidant has been adapted as an assay method for transketolase (see the section on assay methods, this chapter).

Biosynthesis of Transketolase

Lactobacillus viridescens grown on a thiamine-deficient medium showed a sharp decrease in transketolase content, which could be restored in a few minutes when thiamine or thiamine pyrophosphate was added to the growth medium. If harvested deficient cells were incubated for 10 min with cysteine, magnesium, and thiamine pyrophosphate, the transketolase activity was raised to a level twice that of the thiamine-containing control culture. It was concluded that in such cells the apoenzyme was present, and it could bind added thiamine pyrophosphate, forming the holoenzyme when its thiol groups were in a reduced form. In the absence of this reduction, the addition of the coenzyme to an extract of deficient cells could not raise the transketolase activity (Böhm *et al.*, 1973). From experiments with mice treated with thiamine antagonists, it was concluded that in the liver no free apotransketolase was present and that coenzyme incorporation took place at the time the apoenzyme was synthesized (Ostrovskii *et al.*, 1979).

In rye leaves, it was concluded that transketolase was synthesized on cytoplasmic 80 S ribosomes and exported to the chloroplasts (Feierabend and Gringel, 1983).

OTHER ENZYMES

6-Phosphogluconolactonase (EC 3.1.1.31)

The enzyme has been purified to homogeneity from bovine erythrocytes and shown to be a monomer of molecular weight 30,000. It does not require a cofactor and has a specific activity of 26.8 units/mg, corresponding to a turnover number of 858/min. It had a pH optimum of 7.4, for 6-phosphogluconolactone, $K_m = 0.83$ mM at pH 7.4 and 0.7 mM at pH 6.5. The importance of the enzyme in metabolism is that it facilitates the glucose-6-phosphate dehydrogenase reaction by removing its product (Bauer *et al.*, 1983). A K_m of 80 μM for the lactone has been reported for the rat liver enzyme (Schofield and Sols, 1976).

NADPH-GAP Oxidoreductase (EC 1.1.1.177)[1]

$$\text{D-GAP} + \text{NADPH} + \text{H}^+ \longrightarrow \text{GlP} + \text{NADP}^+$$

NADPH-GAP Oxidoreductase, first identified in rat muscle (Wood, 1974c), has been found in other rat tissues (Wood, 1973a; Glushankov *et al.*, 1976) and in mouse peritoneal macrophages (Ravid *et al.*, 1983). It adsorbs to DEAE-Sephadex and can be precipitated by ammonium sulfate. It was stimulated by dithiothreitol and inhibited by *p*-CMB, K_m values were 14 μM and 0.7 μM for D-glyceraldehyde 3-phosphate and NADPH, respectively (Wood, 1973a). D-Glyceraldehyde could replace D-glyceraldehyde phosphate as a substrate at 10-fold higher concentrations (Ravid *et al.*, 1983). An epimerization of either the substrate or the product appears to accompany the reduction reaction. There is some evidence that two enzymes are involved in the reaction as formulated above (T. Wood, unpublished data).

[1] Anonymous (1980).

5

Intermediates in Intact Cells and Tissues

INTRODUCTION

Early methods of measuring pentose phosphate pathway intermediates relied heavily on the use of moderately specific colorimetric procedures, such as the cysteine–carbazole method for ketopentose phosphates and the cysteine–sulfuric acid for heptose phosphates. More recently, relatively specific enzymic or radioisotopic methods have been employed. The use of enzymic methods has depended on the ready availability of purified enzymes, all of which, although expensive, are now commercially available.

Because the enzymes transforming the intermediates of the pathway are present at relatively low activities in most tissues, the quick-freezing techniques developed for the assay of glycolytic intermediates have proved fully adequate. The enzymic measurement of metabolite levels is much more time consuming than assays of enzymes, and, consequently, results of the latter type of measurement predominate. The low levels of the intermediates have encouraged the use of assays in conjunction with a fluorimeter,

rather than with the less sensitive spectrophotometer. The presence of an active pathway in liver has led to the larger number of determinations being carried out on this tissue, and McLean's group, in particular, has studied how changes in dietary and hormonal status of the animal affect metabolite levels in this organ.

LIVER

Measurements of glucose 6-phosphate and 6-phosphogluconate were made on normal rat liver and in hepatomas by Gumaa *et al.*, (1968a), and the work was extended to measurements of pentose phosphates, sedoheptulose 7-phosphate, and erythrose 4-phosphate in liver from rats in a variety of different hormonal and dietary states (Gumaa and McLean, 1968; Greenbaum *et al.*, 1971) (see Table 5.1). Starvation and diabetes decreased the levels of the metabolites studied. Treatment with insulin or refeeding a high carbohydrate diet either restored the levels to those of the control or increased them. Refeeding a high fat diet did not restore the intermediates.

The effect of quinolinic acid, an inhibitor of 2-oxoglutarate transport across the mitochondrial membrane was also investigated (Spydevold *et al.*, 1974), but the only significant difference from the control was a lower level of xylulose 5-phosphate in the treated group of rats. The effect of thiamine deficiency on hepatic metabolites was investigated by McCandless *et al.*, (1975), who found no significant differences between the thiamine-deficient and the control group, despite a reduction of 90% in transketolase activity in thiamine deficiency. Unfortunately, transketolase activity was not measured during the course of the experiments, and the fall was de-

TABLE 5.1

Intermediates of the Pathway in Rat Liver[a,b]

Intermediate	Control	Starved	Starved—refed high carbohydrate diet
G-6-P	178.0 ± 13.0	50.7 ± 2.6	109.0 ± 10.0
6-PG	18.8 ± 2.3	19.8 ± 0.7	24.9 ± 5.0
Pentose-P	429.0 ± 7.2	236.0 ± 12.0	456.0 ± 38.0
S-7-P	25.7 ± 1.4	16.7 ± 2.3	16.1 ± 1.8
GAP	10.6 ± 1.1	16.7 ± 0.8	8.3 ± 1.3
Number of animals	18	6	12

[a] From Gumaa and McLean (1968).

[b] Values are mean ± SEM nmol/g liver.

duced from the results of the authors' previous work. Thyroidectomy was shown to have no significant effect on hepatic metabolite levels in the rat (Baquer *et al.*, 1976), while injection of glucagon, insulin, epinephrine, and ethylaminothiadiazole increased the content of phosphoribosyl pyrophosphate (PRPP) in mouse liver (Lalanne and Henderson, 1975). A careful search led to the identification and estimation of sedoheptulose 1,7-bisphosphate, sedoheptulose 7-phosphate, and octulose mono- and bisphosphates in rat and rabbit liver; however, erythrose 4-phosphate could not be detected (Paoletti *et al.*, 1979b; Horecker *et al.*, 1982). Using a combined enzymic and isotopic assay, Ipata and Camici (1981) found 800 nmol/g of ribose 1-phosphate in rat liver and smaller amounts in brain, kidney, muscle, and spleen, in that order.

BRAIN

In a classic paper, Kauffman *et al.* (1969) described the application of enzymic fluorimetric techniques to the measurement of the metabolites in mouse brain under conditions of ischaemia, anaesthesia, hypothermia, hyperthermia, and the presence of inhibitors. Carl and King (1970) studied the effect of electrically induced convulsions and found that, whereas fructose 6-phosphate, glucose 6-phosphate, and xylulose 5-phosphate fell by comparison with unstimulated animals, 6-phosphogluconate remained constant and fructose bisphosphate levels rose. Kauffman and Harkonnen (1977) measured the intermediates in rat brain synaptosomes and in cerebral cortex and showed that treatment with the antimetabolite 6-aminonicotinamide increased 6-phosphogluconate levels, while the contents of glucose 6-phosphate remained more or less unchanged. The cells convert 6-aminonicotinamide to the 6-amino analog of NADP, which then causes inhibition of glucose 6-phosphate and 6-phosphogluconate dehydrogenases and can also inhibit phosphoglucose isomerase (Herken and Lange, 1969; Lange *et al.*, 1972). From these results it was concluded that the pentose phosphate pathway was active in nerve endings both *in vivo* and *in vitro*. Other studies of 6-phosphogluconate accumulation after treatment with 6-aminonicotinamide have also been carried out on the whole brain (Herken *et al.*, 1969) and on various regions of rat brain (Lange *et al.*, 1970). The administration of the thiamine analog pyrithiamine to mice caused a reduction in brain and liver transketolase activity and an accumulation of 6-phosphogluconate and xylulose 5-phosphate in the brain (Holowach *et al.*, 1968).

ERYTHROCYTES

Early measurements of the intermediates in human erythrocytes were performed by Bucolo and Bartlett (1960), Bartlett and Bucolo (1960), Dische and Igals (1961), and Vanderheiden (1964, 1965). The latter reported the presence of ribose 1,5-bisphosphate, sedoheptulose 1,7 bisphosphate, and octulose 1,8-bisphosphate, and these were later confirmed by Bartlett and Bucolo (1968). Other workers have measured levels of ribose 5-phosphate and phosphoribosyl pyrophosphate (Hershko *et al.*, 1969).

OTHER TISSUES

Lilyblade and Peterson (1962) identified the free sugars sedoheptulose, ribose, and ribulose in postmortem chicken muscle, and Yardley and Godfrey (1963) identified by paper chromatography ribose 5-phosphate, sedoheptulose 7-phosphate, and sedoheptulose 1,7-bisphosphate in extracts of guinea pig skin incubated with glucose and [^{32}P]orthophosphate. The metabolites have been measured in adipose tissue of control and thyroidectomized rats (Baquer *et al.*, 1976) and in mammary gland, slices of mammary tissue, and isolated mammary cells of the rat (Greenbaum *et al.*, 1978). The levels of 6-phosphogluconate have also been determined in the pancreatic islets of fed and starved rats (Hedeskov and Capito, 1975) and in animals injected with 6-aminonicotinamide (Keller *et al.*, 1972). Russian workers have reported on the levels of ketopentose phosphates in the heart muscle, and red and white muscles of five species of Black Sea fish (Kudryavtseva and Zhebentyaeva, 1977). Elution profiles for the column chromatography of ^{14}C-labeled intermediates from rat adipose tissue have been published by Blackmore *et al.* (1982).

ASCITES AND HeLa CELLS

All the intermediates of the pathway have been measured in Ascites tumor cells before and at different times after the addition of glucose to the medium (Gumaa *et al.*, 1968b; Gumaa and McLean, 1969b, 1969c) (see Table 5.2). Experiments were also done to study the effect of oxamate, pyruvate, nicotinamide, and streptozotocin on the accumulation of these intermediates (Gumaa and McLean, 1969a). Up to 200 nmol/ml of cells of ribose 1-phosphate were accumulated by such cells incubated with inosine from an initial level of 15–30 nmol/ml of cells (Barankiewicz and Hender-

TABLE 5.2

Intermediates of the Pathway in Krebs Ascites Cells[a,b]

Intermediate	Endogenous	Endogenous after 10-min incubation with 12.5 m*M* glucose
	Mean ($n = 3-4$)	Mean ± SEM ($n = 6$)
G-6-P	38	444 ± 14
6-PG	8	102 ± 5
Pentose-P	200	2103 ± 78
S-7-P	19	45 ± 5
Er-4-P	5	36 ± 3
PRPP	0	1000
DHAP	214	1033 ± 105
GAP	107	137 ± 13

[a] From Gumaa and McLean (1969b). Reprinted by permission from *Biochem. J.* **115**, 1009–1029. Copyright © 1969. The Biochemical Society, London.

[b] Values are nmol/g cells.

son, 1977). Similar experiments with HeLa cells were carried out by Reitzer *et al.*, (1980), who claimed to have measured 16 μM erythrose 4-phosphate in cells grown on glucose and less than 2 μM in cells grown on galactose.

BACTERIA

A number of workers have reported the presence of seven- and eight-carbon sugars in the cell wall polysaccharides of certain bacteria, and one containing D-xylulose has been found in *Pseudomonas diminuta* (Wilkinson, 1981). A heptose was identified in the cell wall lipopolysaccharide of *Salmonella* (Lehmann *et al.*, 1971), and the same group also reported the presence of 2-keto-3-deoxyoctonate residues (Gmeiner *et al.*, 1971). These compounds were presumably synthesized via enzymes and intermediates of the pentose phosphate pathway, and support for this idea has been provided by the finding that 1-deoxysedoheptulose is accumulated by mutants of *Bacillus pumilus* lacking transketolase (Yokota *et al.*, 1978). It was found that L-*glycero*-D-*manno*-heptose was not synthesized in transketolase-less mutants of *Salmonella typhimurium*, and Eidels and Osborn (1971, 1974) have proposed the scheme shown in Fig. 5.1 for its synthesis.

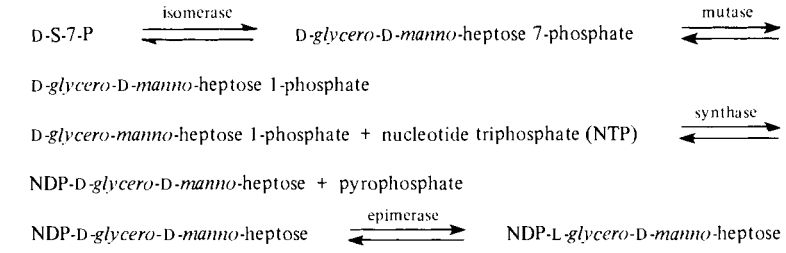


Fig. 5.1. Proposed pathway for the synthesis of nucleotide diphosphate-linked L-*glycero*-D-*manno*-heptose (Eidels and Osborn, 1974).

PLANTS

All the intermediates of the pathway have been measured in *Phaseolus Mungo* seedlings (Ashihara and Komamine, 1974).

INDIVIDUAL INTERMEDIATES

The distribution in nature of heptulose phosphate-forming systems has been reviewed by Nigam *et al.* (1961). Williams *et al.* (1980) have reviewed the reports by various workers of determinations of erythrose 4-phosphate in living organisms and have questioned whether this intermediate of the pathway has ever truly been detected. Using a more specific enzyme assay that they had developed, Paoletti *et al.* (1979c) were unable to detect erythrose 4-phosphate in rat liver or adipose tissue, and it was suggested (Williams *et al.*, 1980) that erythrose 4-phosphate does not occur in a free form in living organisms and that when required it is liberated from sedoheptulose 1,7-bisphosphate by the action of aldolase (see Chapter 2 for further discussion). The detection of this compound and other seven- and eight-carbon sugar phosphates in liver, guinea pig, and erythrocytes has been mentioned earlier in this chapter. A heptitol and an octitol have been identified in the eye lens of man and of the South American rodent *Octodon Degu* by combined gas chromatography-mass spectrometry (Barker *et al.*, 1983).

6

Distribution of the Enzymes among Different Tissues and Different Species

INTRODUCTION

Apart from ribose-5-phosphate ketol-isomerase and ribulose-5-phosphate 3-epimerase, the majority of the enzymes of the pathway have only a low activity in most tissues compared to the enzymes of the glycolytic pathway. Sensitive spectrophotometric and fluorometric procedures are necessary for their measurement, and relatively large amounts of tissue are required for each determination. Most of the enzymes under consideration catalyze reversible reactions, and their rates rapidly decrease as product accumulates, so it is necessary to arrange the assay conditions so that initial velocities are measured as closely as possible.

Although now commercially available, the substrates and auxiliary enzymes required are expensive, and cost may well restrict the number of assays to be performed. Cost limitations may be overcome by the prepara-

tion of one's own substrates and auxiliary enzymes, at the expense of considerable time and trouble. Techniques for the assay of many of the enzymes under consideration have been explored in some detail by Novello and McLean (1968), and most procedures are described in Chapter 4.

Some early investigators used the disappearance of ribose 5-phosphate or the appearance of ketose phosphate as a measure of isomerase activity and the formation of sedoheptulose 7-phosphate as a measure of transketolase activity. These techniques have, however, been superseded by methods that measure the activities of the required enzymes directly and specifically.

The activities of enzymes in a given organ have been expressed in three different ways. The first method gives the total enzyme activity per whole organ, for example, international units per two epididymal fat pads. It is proposed to refer to such values as "content" or "total content." The second method expresses activities as international units per gram of tissue (units/g). These will be referred to as "enzyme levels." The third method quotes values relative to the amount of soluble protein extracted from the tissue, and these will be referred to simply as "specific activities" (units/mg protein). It is worth noting that if the amount of protein extracted per gram of tissue is also given, then specific activities may be converted readily into enzyme levels. Unfortunately, many authors omit this.

By reason of its size and ready availability a majority of measurements have been made on the laboratory rat, while other species have been investigated much less frequently. A considerable body of information on the activities of the enzymes of the pathway in rat tissues under a variety of dietary and hormonal conditions has been provided by McLean and her group, while most other measurements have originated from a variety of laboratories and are scattered through the literature. A survey of the results obtained is given in the next section, according to the species and tissue investigated. Changes in these activities due to experimental manipulation or changes in physiological conditions will be discussed in Chapter 8, and changes in disease and vitamin deficiency are treated in Chapter 10.

GENERAL SURVEYS

Blood and Serum of Different Species

In early investigations by Bruns and his group the levels of isomerase and transketolase were measured in serum and hemolysates of a variety of species, including man. Although the methods used involved the formation of cysteine–carbazole reacting material (ketopentose phosphate) and of a substance reacting with cysteine and sulfuric acid (sedoheptulose 7-phos-

phate), the results clearly show that, among the 11 species examined, the rabbit has the highest isomerase level in its serum at 35.2 μmol/ml/hr of serum and man the lowest at 3.5 μmol/ml/hr of serum. Determinations on sera from a variety of patients showed a pronounced elevation to 11.5 μmol/ml/hr in a lymphogranulomatosis (Bruns, 1956). Measurements on hemolysates showed that, among the 11 species examined, man had the highest isomerase level in the red blood cell at 4.1 mmol/ml hemolysate/hr and chicken the lowest at 0.9 mmol/ml hemolysate/hr (Bruns *et al.*, 1958b).

Measurements of transketolase indicated that, among the 8 species examined, the level within the erythrocyte was between 25 and 95 times higher than that in the serum. The highest whole-blood value was that of the rabbit at 12.8 μmol/ml/hr, and the lowest was the horse at 2.9 μmol/ml/hr. Measurements on blood sera from patients showed a twofold and a threefold elevation over the normal value in virus hepatitis and uremia, respectively (Bruns *et al.*, 1958a).

Muscles of Different Species

All four enzymes of the nonoxidative branch in muscles of a number of species were measured by Tan and Wood (1969), who found particularly low levels of transaldolase in two species of fish. The two dehydrogenases and transketolase were measured in heart and red and white muscles of certain salt water fish, and generally the levels were higher in red muscle and heart than in white muscle (Kudryavtseva and Zhebentyaeva, 1977).

Distribution in Mouse Tissues

One of the earliest systematic investigations of the pathway was that of Glock and McLean (1954), who measured the two dehydrogenases and the rate of ribose 5-phosphate breakdown in mouse skeletal muscle, a sarcoma, and a lymphoma. The two neoplasms had considerably higher enzyme levels than the muscle. The isomerase levels in a number of mouse tissues were measured by Bruns *et al.* (1958b), who found erythrocytes to have a much higher level than heart, skeletal muscle, liver, and kidney. Transketolase levels were in the descending order, liver and kidney, spleen and pancreas, brain and skeletal muscle, and heart (Ostrovskii and Gorenshtein, 1967).

The enzymes in mouse liver and kidney were assayed and compared with the levels in mouse ascites cells and a rat Walker tumor. In all these tissues, transaldolase levels were higher than those of transketolase, and the latter appeared to be the rate-limiting enzyme (Brand *et al.*, 1970c). High levels of transaldolase were determined in mouse liver but that in skeletal muscle

was very low (Hue and Hers, 1974). Transplantable hepatomas of mice were reported to produce a systemic factor that increased the levels of the oxidative branch dehydrogenases in the tissues of the host (Birk and Shapot, 1979).

Distribution in Rat Tissues

The levels of the two dehydrogenases of the pathway and ribose 5-phosphate utilization were studied by Glock and McLean (1954) in liver, blood, heart, and skeletal muscle. Of these liver had the highest and skeletal muscle the lowest levels. All six enzymes of the pathway were measured by Novello and McLean (1968) in liver, adipose tissue, kidney, brain, adrenal gland, lactating mammary gland, and a Novikoff hepatoma. The highest levels were found in lactating mammary gland and the lowest in brain. The enzymes in the hepatoma were present at slightly lower levels than in normal liver. The same enzymes were also measured by Baquer and McLean (1972a), who found that levels of all six enzymes were higher in the hepatoma than in liver. Some typical values are given in Table 6.1.

Transketolase was measured in nine different tissues by Brin (1962), who found the highest level in the liver and the lowest in heart and skeletal muscle. Thiamine deficiency caused transketolase levels to fall in all tissues except brain, and the heart enzyme seemed to be particularly susceptible. Benevenga *et al.*, (1964) pointed out shortcomings in Brin's assays and used an improved method to determine transketolase levels in liver, spleen,

TABLE 6.1

Levels of Pentose Phosphate Enzymes in Rat Tissues[a]

Enzyme	Enzyme level (units/g tissue)[b]			
	Liver	Adipose tissue	Adrenal	Hepatoma
G-6-PDH	1.5 ± 0.1	1.18 ± 0.05	6.4	2.8 ± 0.3
6-PGDH	2.8 ± 0.13	1.0 ± 0.12	7.2	1.3 ± 0.15
Isomerase	3.8 ± 0.2	1.2 ± 0.12	4.1	3.2 ± 0.4
Epimerase	28 ± 2.1	3.9 ± 0.2	31	16 ± 1.4
TK	1.3 ± 0.03	0.4 ± 0.03	1.3	0.7 ± 0.07
TA	1.2 ± 0.04	0.5 ± 0.02	2.7	1.3 ± 0.2
Number of determinations	6	6	2	6

[a] From Novello and McLean (1968). Reprinted by permission from *Biochem. J.*, 107, 775–791. Copyright © 1968. The Biochemical Society, London.

[b] Values are mean ± SEM.

kidney, and brain. Liver gave the highest value and brain the lowest, and, in contrast to Brin's results, the spleen had a higher level than kidney. The effects of changes in diet, hormonal status, and the addition of oxythiamine to the diet were also investigated.

Isomerase levels were determined in 10 different tissues by two independent methods, and two electrophoretic forms of the enzyme were reported in spleen, small intestine, uterus, heart, skeletal muscle, and brain. Spleen had the highest and brain the lowest level (Wood, 1974b).

The NADP-dependent D-glyceraldehyde-3-phosphate oxidoreductase was assayed in 10 tissues by Wood (1973a) and in 3 tissues by Glushankov *et al.* (1976) and found to be highest in skeletal muscle. 6-Phosphogluconolactonase has been assayed in 7 rat tissues and in human erythrocytes (Bauer *et al.*, 1983).

SPECIFIC TISSUES

Rat Liver

It was soon recognized that liver has an active pentose phosphate pathway, and a relatively large number of assays have been carried out on it. The most comprehensive determinations come from McLean's groups, which have determined the enzymes in a variety of dietary and hormonal conditions (Gumaa *et al.*, 1968a, 1969a; Novello *et al.*, 1969). The effects of manganese in the diet (Baquer *et al.*, 1975a), of thyroidectomy (Baquer *et al.*, 1976), and of iron-deficiency anemia (Sochor *et al.*, 1983) were investigated, and measurements have also been carried out on genetically obese rats (Spydevold *et al.*, 1978).

Some early measurements of isomerase, transketolase, and transaldolase were made by Srivastava and Hübscher (1966), and Benevenga *et al.* (1964) investigated changes in the transketolase level resulting from starvation, a high protein, and a high fructose diet and changes in hormone levels. The effects of insulin on transketolase and transaldolase levels (Karaze and Kolotilova, 1973) and the mechanism by which transketolase levels are restored by administration of thiamine to deficient rats (Vinogradov *et al.*, 1979) have been studied. The specific activities of glucose-6-phosphate dehydrogenase and transaldolase in a series of hepatomas (Weber *et al.*, 1974) and in an extract of liver acetone powder (Williams *et al.*, 1978b) have also been reported.

Rat Adipose Tissue and Mammary Gland

The total content of all six enzymes of the pathway in epididymal fat pads in a variety of dietary and hormonal conditions have been determined

(Gumaa *et al.*, 1969b; Spydevold *et al.*, 1978). Changes in the enzyme levels during lipid synthesis (Gumaa *et al.*, 1973) and changes in the levels relative to phosphofructokinase were also studied (McLean *et al.*, 1972).

Rat Brain

The distribution of transketolase in various regions of the brain was determined by Von Bruchhausen (1964). All six enzymes of the pathway have been measured (Kauffman, 1972; Baquer *et al.*, 1973b, 1977) and their levels compared with those of sciatic nerve (Hothersall *et al.*, 1982a). The activities in cerebral cortex and in synaptosomes have also been compared (Kauffman and Harkonnen, 1977). The levels of transketolase, transaldolase, and 6-phosphogluconate dehydrogenase did not change significantly with age, but there was a small and statistically significant decrease in glucose-6-phosphate dehydrogenase (El-Hassan *et al.*, 1981).

Other Rat Tissues

The effect of experimental diabetes on enzyme levels in the kidney (Sochor *et al.*, 1979a, 1979b, 1983) and the influence of iron deficiency on the enzyme profile in adrenal gland (Sochor *et al.*, 1982) have been investigated. The levels of the six enzymes of the pathway in normal and regenerating muscle have been studied by Wagner *et al.* (1978), and revised values for isomerase and epimerase were reported by Wood (1974c). The half-lives of glucose-6-phosphate dehydrogenase and transketolase in heart were measured by Kudryavtseva and Denisova (1980), who concluded that the increases in specific activities brought about by thyroxin were due to increased biosynthesis. Values for the levels of the six enzymes in uterus have been reported by Novello and McLean (1968) and Baquer and McLean (1972b) and for transaldolase by Wood (1974c). The enzymes of the pathway, with the exception of epimerase, have also been measured in intestinal mucosa (Srivastava and Hübscher, 1966).

Ascites and HeLa Cells

All six enzymes of the pathway have been assayed in ascites cells (Tan and Wood, 1969; Gumaa and McLean, 1969b; Brand *et al.*, 1970c) and the two dehydrogenases, transketolase and transaldolase, in HeLa cells (Reitzer *et al.*, 1980).

Subcellular Fractions

The presence of the six pathway enzymes in the large particle fraction of rat tissues and the influence of hormones on their distribution between this

fraction and the cytosol have been studied (Baquer and McLean, 1972a; Baquer *et al.*, 1972). The enzyme levels in rat brain synaptosomes have also been measured (Kauffman and Harkonnen, 1977). The formation of ribose from glucose 6- phosphate by a microsomal fraction of rat liver indicated the presence of the two dehydrogenases, ribose-5-phosphate isomerase, and a phosphatase in this fraction (Hino and Minakami, 1983).

MEASUREMENTS ON OTHER SPECIES

The levels of all six enzymes have been measured in sheep mammary gland (Gumaa *et al.*, 1973) and in extracts of rabbit liver acetone powder (Williams *et al.*, 1978b). Measurements have been made of the enzymes in fish (Mil'man and Yurovitskii, 1969; Kudryavtseva and Zhebentyaeva, 1977) and of transketolase in pigeons (Ostrovskii, 1963; Trebukhina and Ostrovskii, 1964). The levels of transketolase and the two dehydrogenases were determined in pig liver during development (Mersmann and Houk, 1971).

Bacteria, Yeasts, and Fungi

Values for the specific activities of isomerase, epimerase, and transketolase extracted from the parent and a mutant strain of a *Bacillus* species have been published by Sasajima and Yoneda (1974b). Unfortunately, the amount of protein extracted from the cells was not reported, so enzyme levels cannot be calculated. The contents of the four enzymes of the nonoxidative branch in an extract of *E. coli* were measured (Yaphe *et al.*, 1966). The specific activities of all the enzymes of the pathway, with the exception of epimerase, have been reported in *Candida utilis* (Osmond and Ap Rees, 1969) and in *Aspergillus nidulans* (Hankinson and Cove, 1974) and the specific activities and content of transaldolase isozymes in various strains of the former (Araujo Neto and Panek, 1979).

Parasites, Protozoa, and Hydra

All enzymes of the pathway, with the exception of transaldolase, were assayed in *Ascaris lumbricoides* (Entner, 1957), and evidence was produced for the presence of all the pathway enzymes in *Echinococcus granulosus* (Agosin and Aravena, 1960a) and *Ascaris suum* (Langer *et al.*, 1971). All enzymes were measured in certain species of the *Acanthocephala* by Saxon and Dunagan (1975, 1976), and evidence was provided for the presence of all the enzymes except transaldolase in the malarial parasite *Plasmodium*

berghei (Langer *et al.*, 1967). In *Trichomonas vaginalis*, transaldolase activity was high and that of the two dehydrogenases very low (Arese and Cappuccinelli, 1974). The two dehydrogenases have been reported to be absent from some species of hydra (Rutherford and Lemhoff, 1969) and from *Entamoeba histolytica* (Bragg and Reeves, 1962), but Baquer *et al.*, (1975b) have published profiles of the distribution of these enzymes in hydra. In *Entamoeba histolytica* the only pathway enzyme that could be detected was transketolase, and a new metabolic pathway was proposed to operate in this organism (Susskind *et al.*, 1982) (see Chapters 2, 8, and 11).

Plants

All enzymes of the pathway were present in *Phaseolus mungo* seedlings (Ashihara and Komamine, 1974) and, with the exception of epimerase, were measured in *Pisum sativum* (Fowler and Ap Rees, 1970). The two dehydrogenases, transketolase and transaldolase, were assayed in *Acer pseudoplatanus* and shown to be absent from the particulate fraction (Fowler, 1971). The levels of isomerase and ribulose bisphosphate carboxylase in *Hordeum vulgare* were found to change as the light intensity was varied (Huffaker *et al.*, 1966).

CHANGES IN ENZYME LEVELS DURING DEVELOPMENT

Measurements have been made on loach embryos (Mil'man and Yurovitskii, 1969), pig liver (Mersmann and Houk, 1971), and rat brain (Baquer *et al.*, 1973b, 1977) and are discussed in Chapter 8.

DISCUSSION

Assays of the enzymes in a variety of tissues and species indicate that transketolase is the rate-limiting enzyme for the nonoxidative branch of the pathway and either glucose 6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase for the oxidative branch. However, one should bear in mind the reservations expressed in Chapter 9, concerning measurements of transaldolase activity. In rat tissues, enzyme levels are relatively high in adipose tissue, liver, and endocrine glands and low in muscle and brain. The importance of the pathway for brain function has been emphasized, and in muscle the presence of an NADP-specific triose-phosphate oxidoreductase has pointed to a possible modification of the pathway in this tissue (see Chapter 2). The intensive investigations of McLean's group have shown

that the levels of the enzymes in tissues, particularly in liver, adipose tissue, and mammary gland, respond to changes in the diet and are under hormonal control (see Chapter 8).

The presence of the enzymes in a functional state in the particulate fraction from a number of tissues suggests that the requirements for biosynthetic intermediates by mitochondria and cell membranes are specially catered for within the cell. The levels of cytoplasmic enzymes appear to respond to dietary and hormonal influences and increase during growth, development, and the uncoordinated growth of carcinogenesis.

Enzyme levels in microorganisms may be much higher than in animal tissues, and this is illustrated by the wide-spread use of yeasts as a rich starting material for the purification of the various enzymes of the pathway. In some species of parasites and in hydra, certain enzymes of the pathway appear to be missing. More information on the enzymes in such species and the way in which the pathway operates in these organisms would be of great interest.

7

Operation and Regulation in Broken-Cell Preparations

INTRODUCTION

Although earlier workers made extensive use of homogenates and cell extracts to investigate the operation of the pathway, relatively few investigations in recent years have used this approach. A number of different starting substrates have been utilized, and these have been used in the next section as a basis of classification.

THE UTILIZATION OF DIFFERENT SUBSTRATES

Glucose

Apart from the istopically labelled substance (see Chapter 8), glucose has been employed as a substrate only occasionally, probably because control at hexokinase and the dehydrogenase steps prevents the accumulation of high

levels of intermediates. Appel and Parrot (1970) incubated labeled glucose with synaptosomes isolated from rat cerebral cortex and showed that a functioning pentose phosphate pathway was present in these organelles and that its activity was stimulated by neurotransmitters, apparently as the result of a demand for NADPH during removal of the transmitter by monoamine oxidase (Hothersall, *et al.*, 1982b). The accumulation of intermediates of the pathway by a large particle fraction from mouse liver when glucose was added was taken as evidence that a functioning pathway was present (Baquer and McLean, 1972a).

Hexose Monophosphates

Incubation of glucose 6-phosphate with hemolysates of human erythrocytes led to the formation of ribose 5-phosphate, ketopentose phosphates, sedoheptulose 7-phosphate, triose phosphate, and erythrose 4-phosphate (Dische, 1958). A thoroughly dialyzed rat liver supernatant could catalyze the synthesis of sedoheptulose 7-phosphate from fructose 6-phosphate, even in the absence of detectable amounts of acceptor molecules for transketolase and transaldolase. This was explained as being due to a tight coupling of the two enzymes together, such that the product of one enzyme served immediately as the acceptor for the other enzyme (Bonsignore and Grazi, 1965). This coupled reaction was discussed in detail in Chapters 2 and 4.

The action of a rat liver supernatant fraction on mixtures of fructose 6-phosphate and ribose 5-phosphate was investigated by Novello and McLean (1968). They found that the presence of fructose 6-phosphate did not affect the amount of sedoheptulose 7-phosphate formed from ribose 5-phosphate and concluded that fructose 6-phosphate was not a good donor of two-carbon units. Incubation of fructose 6-phosphate with erythrose 4-phosphate, on the other hand, led to considerable sedoheptulose 7-phosphate formation via the transaldolase reaction.

Hue and Hers (1974) incubated labeled glucose 6-phosphate with filtrates of mouse muscle and mouse liver. The liver preparation appeared to act readily via the action of transaldolase on fructose 6-phosphate, whereas in mouse muscle, where the transaldolase level was low, reaction only occurred when both erythrose 4-phosphate and transaldolase were added.

The formation and removal of 6-phosphogluconate when rat liver homogenates and supernatant fractions were incubated with glucose 6-phosphate was investigated and led to the concept that unknown factors present in the tissue reversed the inhibition of the dehydrogenases by NADPH (Eggleston and Krebs, 1974; Krebs and Eggleston, 1974) (see Chapter 9).

Extracts of normal and hypertrophied rat myocardium were incubated with fructose 6-phosphate as a ketose donor and with either glyceraldehyde

3-phosphate or glucose 6-phosphate as acceptors. There was no significant formation of erythrose 4-phosphate and sedoheptulose 7-phosphate in the first case (Stepanova and Severin, 1972), but some erythrose 4-phosphate was formed in the second case (Severin and Stepanova, 1981).

Ribose 5-Phosphate

Most investigators have used ribose 5-phosphate, which is quickly converted into an equilibrium mixture with the two ketopentose phosphates, as a substrate. One of the earliest pieces of work was that of Dickens and Williamson (1956), who investigated its conversion into the other pentose phosphates by extracts of acetone powders of a number of animal tissues. Their results gave a rough indication of the relative activities of isomerase and epimerase in the tissue. In an intensive study, Novello and McLean (1968) followed the formation of sedoheptulose 7-phosphate and triose phosphate by rat liver supernatant fractions and the effect of adding magnesium (required for fructose bisphosphatase activity), fructose 6-phosphate, and erythrose 4-phosphate to the system. The formation of sedoheptulose 7-phosphate by tissue homogenates was used by Dreyfus and Moniz (1962) as a measure of transketolase activity, and they reported that, in rats, white matter had a higher enzyme level than gray matter and that the enzyme level was 70% lower in the brain when the rat was thiamine deficient. The process was not inhibited *in vitro* by the addition of either oxythiamine or neopyrithiamine. The formation of the various intermediates and of hexose monophosphate and glycerophosphate by supernatants from muscle, uterus, and liver of rats was followed by Wood (1974c). In the system containing liver and uterus enzymes, the pathway appeared to operate in the classic manner, but in the muscle system, almost no hexose monophosphates were formed and glycerophosphate accumulated due to the action of an NADP-specific triose-phosphate oxidoreductase (see Chapter 2).

The formation of intermediates from [^{14}C] ribose 5-phosphate by extracts of rat and rabbit liver acetone powders was studied in great detail by Williams and his group under conditions in which further reactions of the hexose phosphate formed were prevented. The distribution of isotope in the hexose phosphate was not as predicted by the classic scheme, and 20% of the substrate carbon could not be accounted for. Substantial amounts of arabinose 5-phosphate and small amounts of *manno*-heptulose 7-phosphate, *altro*-heptulose 1,7-bisphosphate, D-*glycero*-D-*altro*-octulose 1,8-bisphosphate, and D-*glycero*-D-*ido*-octulose 1,8-bisphosphate were reported to have accumulated. When these additional five intermediates were taken into account, 97% of the substrate carbon was accounted for throughout the incubation (Williams *et at.*, 1978a,b). It is noteworthy that, when a specific

assay method for erythrose 4-phosphate was employed, no accumulation of this intermediate could be detected (Williams *et al.*, 1978a; Paoletti, *et al.*, 1974c). In order to account for the previous results a new scheme for the pathway was proposed (see Chapter 2).

The intermediates produced on incubation of an extract of rat adipose tissue with ribose 5-phosphate were studied. Among the products, arabinose 5-phosphate was tentatively identified, but erythrose 4-phosphate appeared to be absent (Blackmore, *et al.*, 1982). Extracts of *Candida guilliermondi* have been shown to incorporate pentose phosphate into the xylene ring of riboflavin during its biosynthesis (Nielsen *et al.*, 1984).

Arabinose 5-Phosphate

The report by Williams's group that D-arabinose 5-phosphate was metabolized by extracts of liver, heart, spleen, skeletal muscle, testis, and pancreas (Blackmore *et al.*, 1971) and could be converted by an extract of rat liver acetone powder to ribose 5-phosphate, hexose monophosphate, and triose phosphate (Williams *et al.*, 1978a) has aroused renewed interest in the metabolism of D-arabinose and its phosphate. Dickens and Williamson (1956) could not detect any formation of pentulose or pentulose phosphate from D-arabinose or D-arabinose 5-phosphate by extracts of acetone powder of a number of tissues. Levin and Racker (1959) found that D-arabinose 5-phosphate was reversibly converted to D-ribose 5-phosphate by extracts of *Pseudomonas aeruginosa,* and a D-phosphoarabinoisomerase has been isolated from *E. coli* (Lim and Cohen, 1966). However, it has been suggested that this enzyme only occurs in bacteria (Lim and Cohen, 1966). Bacterial extracts condensed D-arabinose 5-phosphate and phospho-*enol*-pyruvate to form the 8-phosphorylated derivative of 2-keto-3-deoxyoctonate (Levin and Racker, 1959), which is then incorporated into the cell wall lipopolysaccharide (Lim and Cohen, 1966). Wood and Gascon (1980) were unable to repeat the experiments of Williams *et al.*, (1978a) using extracts of rat muscle, rat liver, and a rat liver acetone powder. No intermediates of the pathway were formed when these extracts were incubated with D-arabinose 5-phosphate, and no formation of arabinose 5-phosphate from ribose 5-phosphate could be detected.

Sedoheptulose Phosphates

No doubt because of their relative expense, few workers have used sedoheptulose compounds as substrates. A mixture of sedoheptulose 7-phosphate and triose phosphate was incubated with a human red blood cell

hemolysate in which 96% of the transaldolase had been inactivated by heating. Erythrose 4-phosphate was found to accumulate but did not do so when a fully active hemolysate was incubated with ribose 5-phosphate (Dische and Igals, 1961). Stepanova (1976) has shown that a cytoplasmic fraction from rat heart muscle cleaved D-sedoheptulose 1,7-bisphosphate, and the formation of sedoheptulose in dialyzed homogenates of rat and bovine tissues was detected. The latter phenomenon appeared to be due to the formation and subsequent hydrolysis of sedoheptulose 7-phosphate from endogenous macromolecular precursors (Hipps, *et al.*, 1981).

Erythrose 4-Phosphate

The synthesis of shikimic acid from D-erythrose 4-phosphate and phospho-*enol*-pyruvate by *E. coli* extracts was demonstrated by Srinivasan *et al.* (1955). The reactions with ribose 5-phosphate and with fructose 6-phosphate catalyzed by transketolase and transaldolase in rat liver supernatants were demonstrated by Novello and McLean (1968). Severin and Stepanova (1973) showed that the aldolase in extracts of rat myocardium could catalyze the rapid condensation of D-erythrose 4-phosphate and dihydroxyacetone phosphate to form sedoheptulose 1,7-bisphosphate.

Inosine and Deoxyinosine

The formation of sedoheptulose 7-phosphate and the 1,7-bisphosphate by human hemolysates acting on inosine was demonstrated by Bucolo and Bartlett (1960), and Lionetti and Fortier (1963) followed the accumulation of hexose and pentose phosphates. Incubation of human red cell ghosts with deoxyinosine led to the formation of D-xylulose 5-phosphate by an unknown mechanism (Lionetti and Fortier, 1966). This mechanism was later shown to involve the production of pentose phosphates from hexosephosphates formed from triose phosphates resulting from the splitting of deoxyribose 5-phosphate by deoxyribose 5-phosphate aldolase (Fortier *et al.*, 1970).

Ribulose 1,5-Bisphosphate

It was observed that human hemolysates acting on ribulose 1,5-bisphosphate or on ribulose 5-phosphate, in the presence of bicarbonate, could form 3-phosphoglyceric acid and pyruvate, apparently due to the presence in the cells of phosphoribulokinase and ribulose-1,5-bisphosphate carboxylase (Fortier, *et al.*, 1967).

D-Arabinose and L-Fucose

Labeled carbon dioxide was produced from these sugars labeled on C-1 by liver homogenates of five animal species, lending support to Williams's contention that D-arabinose can be metabolized by animals (Metzger. *et al.*, 1980).

Proline

Using human erythrocyte extracts as a source of pyrroline-5-carboxylate reductase and pentose phosphate pathway enzymes and using rat kidney mitochondria as a source of proline oxidase, a system was constructed that oxidized [1-^{14}C] glucose and generated ATP when proline was added. It was suggested that in tissues containing proline oxidase, stimulation of glucose oxidation via the oxidative branch of the pathway in the presence of proline could augment the production of ATP (Phang *et al.*, 1980).

8

Operation and Regulation in Intact Cells and Tissues

INTRODUCTION

A very large number of investigators have directed their attention to how the pentose phosphate pathway operates and is controlled in various cells and tissues under a variety of physiological and unphysiological conditions. If it is accepted that the two principal functions of the pathway are to provide NADPH and ribose, then clearly any increases in the demand for these two substances will stimulate the pathway. It is proposed first to consider the various conditions under which activity of the pathway is stimulated and then to look at the effects of changes in the physiological conditions on the pathway. The latter have included hypoxia, high altitude, alterations in hormonal status, pregnancy, muscle regeneration, obesity, changes in diet, neoplasia, normal development, and phagocytosis. The unphysiological conditions have involved stimulation of the oxidative branch of the pathway with methylene blue and phenazine methosulfate, detoxification of drugs, administration of pyruvate, and inhibition of the

pathway by enzyme inhibitors. Some workers have also used mutant strains of organisms lacking one or more enzymes of the pathway to study its function. The operation of the pathway has been reviewed in adipose tissue (McLean *et al.*, 1968), in red blood cells (Dische, 1964), in rat liver (Eggleston and Krebs, 1974; Krebs and Eggleston, 1974), in liver and hepatomas (Weber *et al.*, 1974), in developing brain (Baquer *et al.*, 1977), and in myocardium (Severin and Stepanova, 1981).

Estimation of the Percentage of Glucose Metabolized via the Pathway

There is extensive literature on the methods available for the assessment of the relative contribution of the pentose phosphate pathway and other routes to glucose metabolism. A comprehensive review of this literature will not be attempted here, but more information may be found in the papers of Katz and Wood (1960), Landau *et al.* (1964), Landau and Bartsch (1966), Katz *et al.* (1966), Hostetler and Landau (1967), Katz and Rognstad (1967), Katz and Wals (1972), Baquer *et al.* (1973a), Clark *et al.* (1974), Sernka (1975), Hothersall *et al.* (1979), and Longenecker and Williams (1980a, 1980b) and in the reviews by Katz (1961), Wood *et al.* (1963), and Williams and Clark (1971). The methods involve the measurement of $^{14}CO_2$ formation from [1-^{14}C]glucose and [6-^{14}C]glucose and the incorporation of radioisotopes from specifically labeled substrates into hexose phosphates or other intermediates. A large number of assumptions, conditions, and qualifications have been made in the course of developing these techniques, and Williams and Clark (1971) have suggested that the underlying mechanism assumed for the pathway may itself be incorrect. This question has already been discussed in Chapter 2. Some representative values for the results obtained are presented in Table 8.1. Although the values for a given tissue show wide variations, it is clear that, in rats, the pathway activity is low in muscle and brain, higher in kidney and liver, and probably greatest in adipose tissue, where it is linked to lipid biosynthesis.

Relative Contributions to Pentose Synthesis of the Oxidative and Nonoxidative Branches of the Pathway

Although the pentose phosphate pathway has been regarded as operating as a cycle, it is generally considered as being made up of two separate branches, the activities of which may vary independently. Thus, Novello and McLean (1968), Gumaa *et al.* (1969b), and Baquer *et al.* (1972) found no evidence for the enzymes of the pathway behaving as a "constant proportion group" but rather as an "oxidative group" made up of the two dehy-

TABLE 8.1

Percentage of Glucose Metabolized via the Pentose Phosphate Pathway in Various Tissues

Tissue	Percentage	Reference
Rat adipose	11	Landau and Bartsch (1966)
	11–15	Landau and Katz (1964)
	11–25[a]	Katz *et al.* (1966)
	15–20	Katz and Wals (1971)
	16	Greenbaum *et al.* (1978)
	21–30	Wood *et al.* (1963)
	20–30	Katz and Wals (1972)
	34	Hostetler and Landau (1967)
	42–60	Blackmore *et al.* (1982)
Rat liver	2.8	Hostetler and Landau (1967)
	17	Baquer *et al.* (1973a)
	22–30	Longenecker and Williams (1980b)
Rat kidney	3–5	Hostetler and Landau (1967)
	10–14	Sochor *et al.* (1979a)
Rat adrenal	10.8	Landau and Bartsch (1966)
Rat thyroid	5.3	Landau and Bartsch (1966)
	3.2	Hostetler and Landau (1967)
Rat sciatic nerve	8.3	Hothersall *et al.* (1982a)
Rat brain	0.35[b], 0.8[c]	Hothersall *et al.* (1979)
	1.4	Hothersall *et al.* (1982a)
	2.9	Hosteler and Landau (1967)
Rat muscle	0.8	Hostetler and Landau (1967)
Mouse pancreas	3–4	Hedeskov and Capito (1975)
Monkey brain	5–8	Hostetler *et al.* (1970)
Fetal monkey muscle	<0.4	Beatty *et al.* (1966)
Krebs Ascites cells	1	Brand and Deckner (1970)
	1.2	Gumaa and McLean (1969b)
Bovine erythrocytes	2.7	Love *et al.* (1974)
Human erythrocytes	7[d], 12[e]	Brand *et al.* (1970a)
Bovine adipose	12	Smith (1983)

[a] Value depends on metabolic state.
[b] Cerebral hemispheres.
[c] Cerebellum.
[d] Value determined at pH 7.6.
[e] Value determined at pH 6.8.

drogenases and a "nonoxidative group" made up of transketolase, transaldolase, and the enzymes equilibrating the aldopentose and ketopentose phosphates (Spydevold *et al.*, 1978).

Many attempts have been made to determine the relative contributions of each branch of the pathway to pentose synthesis, and earlier work has been

reviewed by Sable (1966). These investigations have invariably used isotopic tracers, but, as pointed out by Sable (1966), the results must be interpreted with caution, as the contributions to the net synthesis of pentose cannot necessarily be equated with the contributions of the two branches to the labeling of the pentose pool. Thus, Brand and Deckner (1970) concluded that in Ascites tumor cells 20% of the pentose pool was labeled via the oxidative branch and 80% by the nonoxidative branch but that all the net synthesis of nucleic acid pentose occurred by the oxidative branch. Gumaa and McLean (1969b) reached a similar conclusion about the relative contributions of the two pathways in Ascites cells and calculated that 80% of the pentose phosphate pool had been formed by the nonoxidative route. Hofer *et al.* (1971) used *Rhodotorula gracilis,* which does not possess phosphofructokinase, to investigate the problem. They pointed out that in this yeast there was no possibility of fructose 6-phosphate formed via the pentose phosphate pathway being recycled or of dilution of the glyceraldehyde phosphate formed by triose phosphates derived from fructose diphosphate. They found, like Brand and Deckner (1970), almost equal labeling of the pentose pool regardless of whether [1-^{14}C]- or [6-^{14}C]glucose was used as a substrate. They concluded that 80% of the pentose pool was formed by the oxidative branch and 20% by the nonoxidative branch. The evaluation of their results is difficult, however, as they did not indicate the source of the glyceraldehyde phosphate utilized by the nonoxidative branch for pentose synthesis and they found it necessary to postulate a further degradation of the pentose phosphates formed into triose phosphate and a C_2 fragment. Johnson *et al.* (1973) used mutants of *E. coli* deficient in either transketolase or glucose-6-phosphate dehydrogenase and the wild-type organism. They concluded, using ^{18}O-labeled substrates, that pentose was synthesized by both branches of the pathway but that the nonoxidative branch contained only a single transketolase reaction. Their results indicated that fructose 6-phosphate was not the main two-carbon donor for the transketolase reaction, and no definite conclusion was reached as to the relative contributions of each branch of the pathway.

FACTORS STIMULATING THE OXIDATIVE BRANCH OF THE PATHWAY

Methylene Blue and Phenazine Methosulfate

One of the simplest ways to stimulate the oxidative branch of the pathway has been used since the early days of studies on the hexose monophosphate shunt. It involves the reoxidation of NADPH by the addition of a

suitable hydrogen acceptor, such as methylene blue or phenazine methosulfate. When the requirement for reoxidation of NADPH formed by the dehydrogenases is met, it appears that this constraint is removed and the maximal activity of the oxidative branch is revealed. Such an approach has been widely used by McLean and her group (Baquer *et al.*, 1977).

The oxidation of glucose via the shunt in bovine erythrocytes was increased from 2.7% of the glucose metabolized to 14.7% in the presence of methylene blue (Love *et al.*, 1974). In normal fibroblasts, glucose oxidation by the pathway was 0.8% of glucose utilization, which was increased to 18% by methylene blue. In fibroblasts deficient in glucose-6-phosphate dehydrogenase, methylene blue had no effect (Raivio *et al.*, 1981). In Ascites cells, pathway activity was increased by phenazine methosulfate from 1.2% of the glucose used to 11.6% (Gumaa and McLean, 1969b), and in rat adipose tissue the increase was from 15–20% to 30–50% (Katz and Wals, 1971). In the developing chick cornea, pathway activity became detectable in the presence of phenazine methosulfate, even though no activity could be detected in the absence of added acceptor (Masterson *et al.*, 1978). Junge and Brand (1973) studied the stimulation of the shunt activity by the hydroxylation of hexobarbital and showed that the shunt could be further stimulated 330% by the addition of phenazine methosulfate, thus revealing its ultimate capacity.

In rat brain, the activity of the shunt was stimulated 20- to 50-fold by phenazine methosulfate, and it was postulated that this large reserve capacity is connected with the rapid degradation of neurotransmitters and removal of the hydrogen peroxide produced (Baquer *et al.*, 1977; Hothersall *et al.*, 1979).

Pyruvate

The addition of pyruvate is known to stimulate glucose oxidation by certain isolated cells. The effect in Ascites tumor cells (Gumaa and McLean, 1969a), human erythrocytes (Beutler and Guinto, 1974), and cultured rat heart cells (Ravid *et al.*, 1980) appears to be due to reoxidation of NADPH by the cell lactate dehydrogenase.

Drug Detoxification

The pathway may also be stimulated through an increased turnover of NADPH brought about by detoxification processes. Junge and Brand (1973) studied the stimulation of the pathway in isolated rat liver cells by the hydroxylation of hexobarbital and showed that pretreatment of the animals with hexobarbital increased their ability to produce NADPH via the oxida-

tive branch of the pathway. *trans*-Stilbene oxide, a known inducer of drug-metabolizing enzymes increased the cytosolic glucose-6-phosphate dehydrogenase activity by 350% and 6-phosphogluconate dehydrogenase by 170% in rat liver (Seidegaard and DePierre, 1982).

Nitrate and Ammonia Assimilation

When molds such as *Aspergillus nidulans* (Hankinson and Cove, 1974) and *Candida utilis* (Osmond and Ap Rees, 1969) are transferred to a medium rich in nitrate, the enzymes and activity of the pathway increase, apparently in response to an increased demand for NADPH for the reduction of nitrate to ammonium ion. In pea roots, induction of the enzymes of nitrate assimilation was accompanied by a 50% increase in the activity of glucose-6-phosphate dehydrogenase (Emes *et al.*, 1979). By using a yeast mutant lacking the NADP-specific glutamate dehydrogenase, it was shown that the increased shunt activity when ammonium ion was present in the medium resulted from the increased NADP turnover when glutamate was being synthesized (Van de Poll, 1973).

Pyrroline 5-Carboxylic Acid and Proline Synthesis

The reduction of pyrroline 5-carboxylic acid to form proline requires NADPH as a cofactor. The addition of the acid to cultured human fibroblasts stimulated C-1 oxidation via the dehydrogenases of the pathway (Phang, *et al.*, 1979). Fibroblasts from patients with gyrate atrophy lack the ability to synthesize pyrroline 5-carboxylic acid but appear to have the same pentose phosphate pathway activity as fibroblasts from normal individuals (Sandman *et al.*, 1980).

THE EFFECTS OF CHANGES IN PHYSIOLOGICAL FACTORS ON THE PATHWAY

Hypoxia and High Altitude

In rats exposed to 100% oxygen at a pressure corresponding to an altitude of 27,000 ft., the specific activity of hepatic glucose-6-phosphate dehydrogenase increased by half and the activity of the pathway as a whole was doubled, apparently as a result of increased enzyme synthesis (Gorman *et al.*, 1971). A similar increase in the amount of glucose metabolized by the pathway was noted in rat brain exposed to hypoxia (Hakim *et al.*, 1976).

Phagocytosis

Phagocytosis is associated with a burst of metabolic activity that includes increased oxygen consumption, increased H_2O_2 production, and increased activity of the pentose phosphate pathway (Zatti and Rossi, 1965; Klebanoff, 1971, DeChatelet *et al.*, 1972). The microbicidal and tumoricidal properties of phagocytic cells, such as leukocytes and macrophages, appear to depend on the production of "active oxygen" species linked to an increased oxidation of NADPH (Reed, 1969; Badway and Karnovsky, 1980).

It is of interest that the NADPH-glyceraldehyde-phosphate oxidoreductase, first identified in rat muscle (see p. 87), is also present in mouse peritoneal macrophages and may be involved in the regulation of superoxide formation (Ravid *et al.*, 1983).

Nucleic Acid and Protein Synthesis

No correlation was found in isolated mouse pancreatic islets between insulin secretion and flux through the pathway (Hedeskov and Capito, 1975). In rat muscle, regeneration of muscle fibers was accompanied by a ninefold increase in activities of the enzymes of the oxidative branch and smaller increases of the other enzymes. These increases were blocked by inhibitors of RNA and protein synthesis (Wagner *et al.*, 1978). In HeLa cells it was shown that the growth rate was proportional to the rate of pentose phosphate synthesis and that 34% of the pentose phosphate was incorporated into nucleic acids, while the remainder was returned to the glycolysis pathway. The total amount of RNA synthesized was some three to four times the net synthesis, and much of it was unstable and rapidly degraded. It was concluded that in HeLa cells most of the NADPH required could be provided by malic enzyme and that the only essential role of the pathway was to produce pentose for nucleic acid synthesis (Reitzer *et al.*, 1980).

In cultured rat heart muscle cells glucose is known to stimulate protein synthesis under conditions where there is no effect on ATP levels. It was concluded that the restraint on the pathway imposed by the requirement for NADPH reoxidation was removed by the presence of increased amounts of glycolysis and pentose phosphate pathway products, particularly pyruvate and glyceraldehyde phosphate (Ravid *et al.*, 1980). Reoxidation of NADPH was brought about by pyruvate and the nonspecific lactate dehydrogenase and by an NADPH-specific glyceraldehyde-phosphate dehydrogenase (Wood, 1974c).

In rat liver slices the addition of methylene blue increased the availability of ribose 5-phosphate but did not increase purine nucleotide synthesis,

showing that the rate of the pentose phosphate pathway did not limit the rate of this synthesis (Boer *et al.*, 1976).

Fatty Acid Synthesis

In view of the requirements for NADPH in fatty acid synthesis, a number of workers have investigated the operation of the pathway during lipogenesis. In rat liver (Gumaa and McLean, 1971), rat uterus (Baquer and McLean, 1972b), and rat adipose tissue (Katz *et al.*, 1966; Kather *et al.*, 1972a), there was a close correlation between flux through the pathway and fatty acid synthesis.

Glucose-6-phosphate dehydrogenase is considered to be the regulatory enzyme of the oxidative branch of the pathway, the flux being controlled by the amount of enzyme and by the NADPH/NADP ratio (Gumaa and McLean, 1971; Kather *et al.*, 1972b). In developing rat uterus the tissue content of this enzyme increased 5.2-fold when lipid synthesis was stimulated 6.7-fold (Baquer and McLean, 1972b), and in rat mammary gland it increased in parallel with the enzymes of fatty acid synthesis as lactation commenced (McLean *et al.*, 1972). It was calculated that the pathway could provide 80–100% of the NADPH required for fatty acid synthesis in rat mammary gland (Katz and Wals, 1972) and 60% of the NADPH requirement in rat adipose tissue (Kather *et al.*, 1972a). It was concluded that in rat mammary gland the pentose phosphate pathway contributes most of the NADPH required by the tissue, while in sheep mammary gland this function is fulfilled by the cytoplasmic isocitrate dehydrogenase. In rat adipose tissue both the pentose phosphate pathway and malic enzyme provide NADPH (Gumaa *et al.*, 1973).

Diet

Extensive investigations of the effects of changes in diet and hormonal status have been made by McLean and her group and are described in this section. The effects of thiamine deficiency are discussed in Chapter 10.

When rats were starved, the flux through the pentose phosphate pathway in the adipose tissue fell to a low value, and on refeeding it rose to almost twice the normal value (Kather *et al.*, 1972a). In rats refed fat, the flux through the oxidative branch in liver remained low but rose to a value double the control value when they were refed carbohydrate (Gumaa and McLean, 1971). Early work by Benevenga *et al.* (1964) demonstrated that a high-protein or a high-fructose diet, and refeeding starved rats, produced significant increases in the liver transketolase level. This finding was not confirmed by Novello *et al.* (1969), who concluded from their experiments

that 2 days' starvation caused a decrease in 6-phosphogluconate dehydrogenase and epimerase and small increases in transketolase and transaldolase, whether expressed as units/g liver or as specific activities. Refeeding a high-carbohydrate diet restored all levels to normal, except that of glucose-6-phosphate dehydrogenase, which showed "overshoot" to values almost three times normal. Refeeding a high-fat diet restored all levels to approximately normal.

Similar experiments in rat adipose tissue showed that 2 days' starvation caused the specific activities of isomerase and epimerase to fall significantly. Refeeding a high-carbohydrate or high-fat diet restored the specific activities of all enzymes to normal, except for transketolase, which overshot to above normal values in rats refed carbohydrate. Starvation for a further day brought about a further marked decrease in the total contents of glucose 6-phosphate dehydrogenase and transketolase (Gumaa *et al.*, 1969b). In rat liver, starvation brought about a marked fall in the levels of glucose 6-phosphate, pentose phosphates, and sedoheptulose 7-phosphate, and refeeding a high-fat diet caused the level of 6-phosphogluconate to fall and the pentose phosphate levels to decrease even further. Refeeding a high-carbohydrate diet restored the levels of intermediates more or less to normal (Gumaa and McLean, 1968; Greenbaum *et al.*, 1971).

From the results of these and similar experiments, the construction of "cross-over plots," and the consideration of equilibrium constants for the enzymes of the pathway and mass action ratios, Gumaa and McLean (1971) concluded that, in rat liver at least, the rate of the pathway was controlled by glucose-6-phosphate dehydrogenase and the NADPH/NADP ratio. The identification of the site of control at the steps catalyzed by the two dehydrogenases is supported by the finding that the rate of synthesis of liver 6-phosphogluconate dehydrogenase was markedly increased in rats fed a high-carbohydrate diet and that the level of the enzyme in the tissue was governed by the rate of synthesis (Procsal *et al.*, 1976). The increase in glucose-6-phosphate dehydrogenase levels in the liver brought about by a high-carbohydrate diet or by thyroid hormone was accompanied by an increase of the corresponding messenger RNA, and this increase was specific to liver among the tissues examined (Miksicek and Towles, 1983).

When reviewing the previous results, it should be borne in mind, as pointed out by Weber and Hird Convey (1966), that the effects of starvation may be considered as a response to a low level of circulating insulin and of refeeding as due to the stimulation of insulin secretion.

The enzyme profiles of genetically obese rats given food *ad libitum* were compared with those of their lean litter mates by Spydevold *et al.* (1978), who found that the levels of the oxidative enzymes of the pathway in liver were approximately doubled, while the nonoxidative enzymes remained

about the same. In adipose tissue, the total content of all the enzymes of the pathway were higher. When the food intake of obese rats was restricted and they were compared with free-fed obese rats, the only significant change in liver was an increase in transketolase, expressed as units/mg DNA. In adipose tissue, on the other hand, the nonoxidative enzymes increased and the two dehydrogenases decreased. This was interpreted as an adaptive response coordinated with depressed RNA synthesis and increased degradation of pentose phosphates in a condition of food limitation. These changes in opposite directions demonstrate how the two segments of the pentose phosphate pathway do not always maintain constant proportionality.

It has been shown that iron deficiency and the addition of iron and manganese to the diet of newly weaned rats may cause pronounced changes in the enzymes of the pathway and in the conversion of glucose to lipid in the liver, adipose tissue, and adrenal glands. These effects may be secondary to changes in thyroid function (Baquer *et al.*, 1975a, 1982; Sochor *et al.*, 1982).

Diabetes, Insulin, and Glucagon

In rat adipose tissue the percentage of glucose metabolized by the pentose phosphate pathway was increased from the range 11–15% to 23–25% in the presence of insulin (Landau and Katz, 1964; Landau and Bartsch, 1966; Katz *et al.*, 1966). Kather *et al.* (1972a) found an increase from 7.7 to 12.2% in adipose tissue when rats were treated with insulin and an increase to 18.4% on insulin treatment of rats previously starved and refed. In rat liver, Gumaa and McLean (1971) concluded that the flux through the pathway was governed by the rate of lipogenesis. In diabetes, lipid synthesis and flux through the pathway were decreased, but restored to levels approaching normal by treatment with insulin. A similar result was obtained with isolated adipocytes from rat mammary gland (Greenbaum *et al.*, 1978). Insulin caused increases in both the flux through the oxidative branch of the pathway and lipid synthesis in adipose tissue of genetically obese rats, both limit fed and fed *ad libitum*, and in their lean litter mates (Spydevold *et al.*, 1978). An increased flux of glucose through the pathway and an elevated level of the enzymes was found in diabetic rat kidney when compared to that of the normal animal, and this was considered as evidence of glucose overutilization in this organ (Sochor *et al.*, 1979a,b).

Diabetes caused by the administration of alloxan to rats increased the specific activities of transketolase and transaldolase in adipose tissue (Gumaa *et al.*, 1969b), and in liver, decreased the levels of 6-phosphogluconate dehydrogenase, isomerase, epimerase, and transketolase, without

affecting glucose-6-phosphate dehydrogenase or transaldolase (Novello *et al.*, 1969).

Treatment of diabetic rats with insulin increased the specific activities of all the enzymes above the normal values in adipose tissue, while glucagon had no effect (Gumaa *et al.*, 1969b). In the liver, all the enzymes were increased to above normal levels except epimerase, which remained low (Novello *et al.*, 1969). In rat liver, insulin was shown to induce the synthesis of glucose-6-phosphate dehydrogenase in a dose-responsive way, and the level of the enzyme was proportional to the level of circulating insulin (Weber and Hird Convey, 1966; Gumaa and McLean, 1971; Winberry *et al.*, 1980). Transketolase- and transaldolase-specific activities were decreased in rat liver by diabetes and starvation for 3 days. When insulin was injected, the transketolase activity increased in normal, diabetic, and starved rats but transaldolase activity increased significantly only in normal rats (Karaze and Kolotilova, 1973).

The levels of the intermediates in the livers of rats made diabetic with alloxan were measured on two separate occasions by McLean's groups (Gumaa and McLean, 1968; Greenbaum *et al.*, 1971). There was little agreement between the two sets of values as to the effects of diabetes and of subsequent treatment with insulin. The only consistent finding was a fall in the level of pentose phosphates in the diabetic rat and a rise to normal or above normal values when insulin was administered. In perfused rat livers, glucagon increased the level of sedoheptulose 7-phosphate fivefold and markedly decreased the level of sedoheptulose 1,7-bisphosphate. It was suggested that these changes were mediated via the action of fructose 2,6-bisphosphate on phosphofructokinase (Blackmore and Shuman, 1982). Glucagon had no effect on the rate of synthesis of 6-phosphogluconate dehydrogenase in rat liver (Procsal *et al.*, 1976).

In alloxan diabetes, the flux of glucose through the pentose phosphate pathway was decreased by 70% in the rat heart, in parallel with a similar decreased flux via the glycolytic pathway. Both effects were believed to be the result of a fall in hexokinase activity. An additional factor may be a fall in the fructose 2,6-bisphosphate level (Sochor *et al.*, 1984).

Other Hormonal Effects

Pregnancy and Lactation

Specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and the rate of fatty acid synthesis in rat liver and adipose tissue were almost doubled when rats became pregnant (Herrera and Knopp, 1972). In mammary gland at the onset of lactation only

glucose-6-phosphate dehydrogenase showed an increase when the levels of the enzymes of the pathway were related to the levels of phosphofructokinase (McLean *et al.*, 1972). Progesterone produced a dose-dependent inhibition of the oxidative pathway in this gland, and oxytocin caused a large stimulation (Greenbaum *et al.*, 1978). Immature rat uterus treated with oestradiol showed a marked increase in the total content of glucose-6-phosphate dehydrogenase and isomerase compared to the controls (Baquer and McLean, 1972b).

Adrenal Glands

Adrenocorticotropin had no effect on the percentage of glucose metabolized by the pathway in rat adrenal glands (Landau and Bartsch, 1966). Adrenalectomy was reported to decrease the transketolase and epimerase levels in rat liver, and the effect was reversed by cortisone and hydrocortisone (Benevenga *et al.*, 1964; Novello *et al.*, 1969). The total contents of all the enzymes of the pathway in rat adipose tissue were reduced by adrenalectomy but were not restored by cortisone (Gumaa *et al.*, 1969b). Corticosterone inhibited the activity of the oxidative branch of the pathway in rat mammary gland, but hydrocortisone had no significant effect (Greenbaum *et al.*, 1978).

Thyroid

Feeding iodinated casein or administration of thyroxine to rats had no effect on the liver transketolase level (Benevenga *et al.*, 1964). Thyrotropin caused an increase in the percentage of glucose metabolized by the pathway in the thyroid (Landau and Bartsch, 1966). Thyroidectomy did not change this percentage in rat liver, nor did it significantly alter the levels of the enzymes or intermediates of the pathway (Baquer *et al.*, 1976), although earlier a decrease in the levels of all enzymes of the pathway except transaldolase had been reported (Gumaa *et al.*, 1969b). In rat adipose tissue, a decrease in the specific activities of all the enzymes of the pathway was found (Gumaa *et al.*, 1969b).

Pituitary

Hypophysectomy lowered transketolase levels in rat liver, and they were restored by thyroxine and by growth hormone (Benevenga *et al.*, 1964; Novello *et al.*, 1969). Growth hormone caused a small but significant drop in the level of rat liver ransketolase (Gumaa *et al.*, 1969a) but had no apparent effect on the percentage of glucose metabolized by the pathway in rat adipose tissue (Landau and Bartsch, 1966).

Development and Neoplasia

Changes in the pentose phosphate pathway in brain during development are discussed in the section devoted to that tissue (see later this chapter). The oxidative branch of the pathway had greater activity in muscle from fetal monkeys than from infant monkeys (Beatty *et al.*, 1966). The regeneration of muscle fibers in rats treated with Marcaine (1-*n*-butyl-DL-piperidine-2-carboxylic acid 2,6-dimethylanilide hydrochloride) was accompanied by a very marked increase in the specific activities of the two dehydrogenases and a lesser increase in the other enzymes of the pathway. These changes appeared to be the result of increased synthesis of the enzymes (Wagner *et al.*, 1978). In myocardial hypertrophy, however, the transketolase level decreased by 70% (Clark *et al.*, 1972b). In the liver of developing pigs, the transketolase level increased as the animal matured, whereas glucose-6-phosphate dehydrogenase did not change (Mersmann and Houk, 1971).

In developing chick cornea the appearance of pentose phosphate pathway activity coincided with the onset of corneal transparency (Masterson *et al.*, 1978). During development of the embryo of the loach only glucose-6-phosphate dehydrogenase and fructose bisphosphatase changed in total content during development, both declining as the number of cell divisions declined (Mil'man and Yurovitskii, 1969). In tissue culture, interferon blocked the differentiation of a preadipose cell line, apparently by inhibition of the pentose phosphate pathway and the associated reduction of thiol groups necessary for differentiation (Saneto and Johnson, 1982).

A number of workers have used the ability of malignantly transformed cells to grow freely either in tissue culture or *in situ* to investigate the operation of the pathway in such cells. Thus, McLean's groups have looked at the levels of enzymes and intermediates in Krebs Ascites cells and the ways in which they change under various conditions. It was concluded that in such cells glucose-6-phosphate dehydrogenase and transketolase played a regulatory role and that 20% of the pentose phosphate was formed by the oxidative branch and 80% by the nonoxidative branch of the pathway (Gumaa *et al.*, 1968b; Gumaa and McLean, 1969a,b). In HeLa cells the cellular growth rate was proportional to the rate of pentose phosphate synthesis. It was concluded that 34% of the pentose phosphate synthesized by the oxidative branch was used for nucleic acid synthesis and that the only essential function of the pathway in these cells was pentose phosphate production rather than the formation of NADPH (Reitzer *et al.*, 1980).

In Novikoff and ethionine-induced hepatomas the glucose-6-phosphate dehydrogenase level was much higher than in normal rat liver (Gumaa *et al.*, 1968a). In a series of rat hepatomas of gradually increasing growth rate, the specific activities of glucose-6-phosphate dehydrogenase and transal-

dolase increased in step with the growth rate, and they were identified as the rate-limiting enzymes in these tissues. It was concluded that in normal and diabetic rat liver the two dehydrogenases, transketolase and transaldolase, were controlled genetically together as a group (pleiotropic control) (Weber *et al.*, 1974). From investigations of a series of mouse hepatomas, it was claimed that a systemic factor produced by the neoplasm was responsible for inducing increases in the levels of transketolase and the two dehydrogenases in the tissues of the host (Birk and Shapot, 1979).

Operation of the Pathway in Brain

This topic has been most ably reviewed by Baquer *et al.* (1977). In 1962, Hotta showed that the oxidation of [1-^{14}C]glucose by minced guinea pig brain was stimulated by the addition of both NADP and oxidized glutathione (GSSG), and he concluded that the pathway played an important role in reducing glutathione and maintaining the intracellular environment in a reduced state. O'Neill and Duffy (1966) showed that the oxidative branch of the pathway was more than twice as active in newborn dog brain than in adult brain and that the pathway possessed a very large reserve capacity, which was revealed in the presence of phenazine methosulfate. The percentage of glucose metabolized by the pentose phosphate pathway was calculated as 2.3% (Gaitonde *et al.*, 1983), 2.9% in rat brain (Hostetler and Landau, 1967), and 5–8% in monkey brain (Hostetler *et al.*, 1970). As rat brain developed over the period 1–20 days after birth and then to adulthood, the percentage of glucose metabolized by the pathway decreased steadily (Hothersall *et al.*, 1979). In hypoxia, more glucose was metabolized via the pathway than normal, but the reason for this increase was not identified (Hakim *et al.*, 1976).

In young rats undernourished by restricted feeding, the flux through the oxidative branch in the brain was increased, and it returned to normal on normal feeding. The levels of the two dehydrogenases in the brain were lower at 10 days and higher at 18 days than in control animals. These differences disappeared when the tissue was homogenized in the presence of Triton X-100, and it was suggested that in undernourished animals less enzyme was bound in the particulate fraction of the tissue (Shankar *et al.*, 1983).

In guinea pig brain, changes in the degree of reduction of glutathione produced by drugs was paralleled by changes in activity of the oxidative branch of the pathway, and it was concluded that the pathway played a major role in brain in maintaining glutathione in the reduced state (GSH) (Hotta and Seventko, 1968). From their experiments, Vallejo *et al.* (1971) concluded that the main factor controlling the pathway in brain was the

NADPH/NADP ratio. The enzymes of the pathway in rat brain were assayed during development. They remained relatively constant in content except for transaldolase which fell progressively from age 1 day to adulthood, and transketolase, which increased steadily over the same period (Baquer *et al.*, 1973b, 1977). As rats aged, the level of glucose-6-phosphate dehydrogenase in the brain increased significantly over the period 20 days to 24 months, thus demonstrating that they do not lose the capacity to generate NADPH in this tissue (El-Hassan *et al.*, 1981).

The activity of the pathway in brain slices fell rapidly with the age of the rats from 20 days on, until no activity could be detected at 18 months. However, the fully stimulated activity obtained by treatment with phenazine methosulfate only declined after 23 months. The fall in the manifest activity of the pathway was attributed to decreased NADPH turnover as a result of a decreased synthesis of fat, glutamate, and γ-aminobutyric acid. The pathway was also stimulated by 5-hydroxytryptamine, and the degree of stimulation increased with age (Zubairu *et al.*, 1983).

Appel and Parrot (1970) showed that in synaptosomes isolated from rat cerebral cortex, an active pentose phosphate pathway was present and was stimulated by neurotransmitters. The pathway in rat cerebral cortex slices was activated by electrical stimulation of the slices, and it was concluded that the pathway plays an important role in the functioning of synapses (Kimura *et al.*, 1974). Neurotransmitters stimulated the oxidative branch of the pathway in synaptosomes, and the stimulation was prevented by inhibitors of monoamine oxidase. It was concluded that the function of the pathway and its large reserve capacity in synaptosomes was to protect them against oxidation and to remove the hydrogen peroxide produced by the action of monoamine oxidase on transmitters released during neural function (Baquer *et al.*, 1977; Hothersall *et al.*, 1982b). Treatment of rats with 6-aminonicotinamide produced neurological disorders, apparently resulting from an impaired operation of the pathway in brain (Gaitonde *et al.*, 1983).

Operation and Control in Different Species

Most investigations of the pathway have been carried out using mammalian tissues, bacteria, and yeasts. However, a few workers have concerned themselves with other species that are briefly described in this section. The enzymes and characteristic sugars of the pathway were all detected in *Echinococcus granulosus*, and it was shown that glucose 6-phosphate and lactate could be formed from ribose 5-phosphate. No evidence of isomerization of D-arabinose or D-xylose could be detected in this organism (Agosin and Aravena, 1960a). In the parasitic amoeba *Entamoeba histolytica*, glu-

cose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and transaldolase appeared to be absent. However, there was a pathway for the conversion of hexose to pentose, which was believed to use aldolase, transketolase, and a pyrophosphate-dependent phosphofructokinase (Susskind *et al.*, 1982). In hydra, there was a gradient of the specific activities of the two dehydrogenases of the pathway from foot to head, and it was suggested that these gave positional information to the organism during development (Baquer *et al.*, 1975b). Rutherford and Lemhoff (1969), however, reported that 6-phosphogluconate dehydrogenase was absent from certain species of hydra, and the 6-phosphogluconate that accumulated as a result was removed by phosphatase hydrolysis. In the loach, of the enzymes of the pathway only glucose-6-phosphate dehydrogenase and fructose bisphosphatase were observed to change during development (Mil'man and Yurovitskii, 1969).

Apart from investigations of the reductive pentose phosphate pathway not discussed here, relatively few studies have been made of the operation of the pathway in plants. Some studies on plants, fungi, and yeasts have been discussed in the previous section on nitrate and ammonia assimilation. The relative importance of glycolysis and the pentose phosphate pathway was estimated by tracer methods and from measurements of their characteristic enzymes in segments of differentiating pea roots. Glycolysis was predominant at the growing tip, but the pentose phosphate pathway increased relatively to glycolysis as the root became more differentiated (Fowler and Ap Rees, 1970). The distribution of enzymes and intermediates of the pathway in the hypocotyls of dark-grown seedlings of *Phaseolus mungo* was measured and mass action ratios calculated. Glucose-6-phosphate dehydrogenase and transketolase were identified as regulatory enzymes. Pathway activity was greatest at the growing tip, declining as the distance from the tip increased (Ashihara and Komamine, 1974).

Work with some other species is also described later in the section treating studies on mutant strains.

The Effects of Enzyme Inhibitors on the Pathway

Very few effective inhibitors of enzymes of the pathway are known, so only a few studies of the results of inhibiting the pathway have been made. The use of thiamine analogs to bring about inhibition at the transketolase steps is discussed together with the effects of thiamine deficiency in Chapter 10.

Aminonicotinamide was introduced as an inhibitor of 6-phosphogluconate dehydrogenase by Herken and Lange (1969), and attempts to inhibit the oxidative branch employ this compound. It is converted into a 6-aminoni-

cotinamide analog of NADP that inhibits 6-phosphogluconate dehydrogenase and causes 6-phosphogluconate to accumulate (Lange *et al.*, 1970). This latter compound, in turn, inhibits phosphoglucoisomerase (Herken *et al.*, 1969; Lange *et al.*, 1972), producing a secondary effect of blocking glycolysis. Aminonicotinamide caused a large increase in 6-phosphogluconate levels in rat brain (Herken *et al.*, 1969), rat kidney (Kolbe *et al.*, 1971), and rat adipose tissue (Kather *et al.*, 1972b). It was pointed out, however, that an accumulation of 6-phosphogluconate does not necessarily imply a decreased flux through the oxidative branch. In rat adipose tissue, 6-phosphogluconate dehydrogenase was not the rate-limiting enzyme, and the flow through the pentose phosphate pathway, the rate of fatty acid synthesis, and the ability to be stimulated by insulin were all unaffected by 6-aminonicotinamide (Kather *et al.*, 1972b). In bovine adipose tissue, lipogenesis and NADPH production were even stimulated by the presence of the inhibitor (Smith and Prior, 1984).

Rat brain is particularly sensitive to this inhibitor, which appears to affect glial cells more than neurons (Kolbe *et al.*, 1976). There appears to be a hierarchy of response, with the pentose phosphate pathway being the most sensitive, followed by glycolysis and fatty acid formation, then the pyruvate dehydrogenase reaction, the glutamate–γ-aminobutyrate route, and, finally, the tricarboxylic acid cycle (Hothersall *et al.*, 1981). In human fibroblasts, the oxidative branch was 86% inhibited by aminonicotinamide, yet there was no significant change in the levels of ribose 5-phosphate or phosphoribosyl pyrophosphate (PRPP), and it was concluded that pentose phosphate for nucleotide synthesis was supplied predominantly by the nonoxidative branch of the pathway (Raivio *et al.*, 1981).

The indirect effects of inhibitors that slowed the turnover of NADP and NAD were investigated by Gumaa and McLean (1969a). They concluded from changes in the levels of intermediates of the pathway that, in Ascites cells, the pathway operated as two independent branches. Quinolinic acid, an inhibitor of the transport of 2-oxoglutarate across the mitochondrial membrane, was observed to significantly decrease the level of xylulose 5-phosphate in rat liver; however, no comment was made on this finding (Spydevold *et al.*, 1974). Many enzymes of the pathway are inhibited by inorganic phosphate, and the effect of high concentrations of this ion on glucose oxidation by human red blood cells was attributed to the inhibition of transketolase and transaldolase (Sagone *et al.*, 1972).

Studies with Mutant Strains

Mutant strains of *E. coli* lacking either glucose-6-phosphate dehydrogenase or transketolase were employed to investigate the mode of synthesis of

ribose from [^{18}O]glucose and [^{18}O]fructose. Growth of the mutant lacking transketolase on [^{18}O]glucose yielded unlabeled pentose phosphate, showing that there was no equilibration of the C-1 oxygen and the C-6 oxygen of glucose via the triose phosphates. When a mutant lacking the dehydrogenase was used, nearly 50% of the ^{18}O of the substrate appeared on the oxygen atom at C-5 of the pentose phosphate, suggesting that the nonoxidative branch consisted of a single transketolase reaction, rather than a combination of two transketolase and one transaldolase reactions (Johnson *et al.*, 1973). When an *E. coli* mutant containing a 10-fold higher level of glucose-6-phosphate dehydrogenase than normal was studied, the *in vivo* rate of glucose oxidation was only 40% higher than in normal cells. It was concluded that either the activities of the dehydrogenases were regulated by hitherto unidentified metabolites or that their kinetic properties within the cell were unlike those determined *in vitro* (Orthner and Pizer, 1974).

Studies of mutants of *Drosophila melanogaster* showed that the absence of glucose-6-phosphate dehydrogenase had no effect on fertility or viability of the flies but that an absence of 6-phosphogluconate dehydrogenase was lethal. When both enzymes were missing the lethal effect disappeared (Gvozdev *et al.*, 1976). The authors concluded that in *Drosophila* the oxidative branch of the pathway was not essential for NADPH production, and, in a review of the subject, Lucchesi *et al.* (1979) have suggested that the lethal effect of 6-phosphogluconate dehydrogenase deficiency is due to the toxicity of the 6-phosphogluconate that accumulates. A mutant of *E. coli* lacking 6-phosphogluconolactonase was found to grow only slightly more slowly than the wild strain, and it appeared that the lactonase was not essential for the hydrolysis of 6-phosphogluconolactone. In a strain lacking phosphoglucoisomerase as well, growth occurred on glucose but at a very slow rate, indicating that the pentose phosphate pathway could serve as the sole route for glucose catabolism (Kupor and Fraenkel, 1972).

Morgan (1981) used a strain of cultured Chinese Hamster cells, deficient in phosphoglucoisomerase, to test the suggestion of Williams *et al.* (1978a) and of Severin and Stepanova (1981) that the pentose phosphate pathway could operate in such a way as to produce glucose 6-phosphate directly from pentose phosphate without first producing fructose 6-phosphate. The cells did not grow on ribose alone unless they were supplemented by phosphoglucoisomerase, thus supporting the classic formulation of the pathway and disproving the previous types of alternative. Similarly, a mutant of *Saccharomyces carlsbergensis* lacking the NADP-dependent glutamate dehydrogenase was used to show that this enzyme was responsible for increased activity of the oxidative branch of the pathway when ammonia was being assimilated (Van de Poll, 1973).

Sasajima and his group have made extensive investigations of mutants of a *Bacillus* species lacking epimerase or transketolase (Sasajima and Yoneda, 1971, 1974b). As expected, mutants lacking transketolase could not assimilate gluconate or pentoses and required shikimic acid for the synthesis of aromatic acids (Sasajima and Yoneda, 1974b; Sasajima *et al.*, 1977). An unexpected observation was that in such mutants the assimilation of glucose was impaired (Sasajima *et al.*, 1977). The defect in glucose assimilation by *Bacillus subtilis* mutants lacking transketolase was traced to alterations of the cell surface and a defective phospho-*enol*-pyruvate-dependent glucose transport system. It was suggested that these changes were the result of a disorder in the synthesis of ribitol-teichoic acid required for construction of the cellular membrane (Sasajima and Kumada, 1979, 1981a, 1981b). The mutants lacking transketolase were shown also to be lacking flagellae (Sasajima and Kumada, 1983a, 1983b). A mutant of *Bacillus pumilus* containing very low levels of transketolase was shown to accumulate 1-deoxy-D-*altro*-heptulose (1-deoxysedoheptulose) and the corresponding 2,7-anhydride (1-deoxysedoheptulosan) (Yokota *et al.*,1978), and a 1-deoxy-D-*altro*-heptulose phosphate synthase has been partially purified from extracts of the mutant. This enzyme could synthesize the deoxy compound from DL-acetoin and D-ribose 5-phosphate in the presence of Mg^{2+} and thiamine pyrophosphate (TPP) (Yokota and Sasajima, 1983). A transketolase-deficient mutant of *Salmonella typhimurium* was unable to synthesize sedoheptulose 7-phosphate and produced an incomplete heptose-deficient lipopolysaccharide (Eidels and Osborn, 1974).

9

Overall Control of the Pathway

INTRODUCTION

A number of different approaches are possible when seeking to identify the manner in which a metabolic pathway is controlled (Newsholme and Start, 1973). They are as follows:

I. Identification of the rate-limiting enzyme of the pathway. If this is also a regulatory enzyme its activity will be controlled in an independent manner by biosynthesis, allosteric activation or inhibition, or conversion from or to a form of different specific activity.

II. Comparison of mass action ratios in the "steady state" with the corresponding apparent equilibrium constant for each enzyme of the pathway. Reactions far removed from equilibrium usually indicate that the enzyme responsible is being controlled in some way.

III. Measurements of the steady-state concentrations of intermediates and the construction of cross-over plots when the flux through the pathway is altered (other than by changing the input or output of material) and the system changes from one steady state to another.

IV. The identification of enzymes under hormonal and/or allosteric control.

V. The identification of enzymes that change in amount or specific activity as the physiological environment changes.

The application of these ideas to the pentose phosphate pathway is complicated by uncertainties as to whether the pathway should be considered as a shunt starting with glucose 6-phosphate and ending with the reentry of fructose 6-phosphate and triose phosphate to the glycolysis pathway or whether it should be considered as two separate pathways, the oxidative branch and the nonoxidative branch, leading to the formation of ribose 5-phosphate. An additional complication is the possibility, discussed in Chapter 2, that transketolase and transaldolase are tightly coupled together and function as a unit. Assays of the two enzymes individually then might not give a true picture of their combined activity. Most assays of transaldolase have been made in the reverse direction (fructose 6-phosphate + erythrose 4-phosphate → sedoheptulose 7-phosphate + glyceraldehyde phosphate), whereas transketolase has usually been measured, in tissues, in the forward direction (ribose 5-phosphate + xylulose 5-phosphate → sedoheptulose 7-phosphate + glyceraldehyde phosphate). Wood has shown that the maximal velocity of transaldolase that can be measured in the forward direction (sedoheptulose 7-phosphate + glyceraldehyde phosphate → fructose 6-phosphate + erythrose 4-phosphate) is only one-third that of the reverse direction, the one which is usually measured (Wood, 1972). Dividing the measured values for transaldolase activity by a factor of three will, in most tissues, change the identification of the rate-limiting enzyme for the nonoxidative branch from transketolase to transaldolase.

The use of mass action ratios and cross-over plots when erythrose 4-phosphate is one of the intermediates involved is complicated by the difficulties in measuring accurately the small amounts of this compound that accumulate in biological systems and by suggestions that this intermediate has never convincingly been detected in such systems (Williams *et al.*, 1980).

With the previous reservations in mind, it is proposed to look at how various groups of workers have applied the approaches listed to the identification of the factors controlling the pathway.

APPROACH I: RATE-LIMITING ENZYMES

Novello and McLean (1968) assayed all the enzymes of the pathway in six different tissues of the rat and in a hepatoma and concluded that transketolase was the rate-limiting enzyme but that the oxidative branch was

controlled by the dehydrogenases and by the rate of reoxidation of NADPH. In rat adipose tissue, a similar conclusion was reached, that the rate of the oxidative branch was controlled by the rate of NADPH regeneration by lipid biosynthesis, and it was deduced that 6-phosphogluconate dehydrogenase was not rate limiting in this tissue (Kather *et al.*, 1972b). Eggleston and Krebs (1974)(Krebs and Eggleston, 1974) concluded that in rat liver there was a coarse long-term control of the oxidative enzymes of the pathway by enzyme induction on feeding and a finer short-term control by the NADPH/NADP ratio and "deinhibition" of glucose-6-phosphate dehydrogenase. This idea is discussed in more detail later. It was concluded by Weber *et al.* (1974), on rather inadequate experimental evidence, that in rat liver and in hepatomas glucose-6-phosphate dehydrogenase and transaldolase were the rate-limiting enzymes and the two dehydrogenases, transketolase, and transaldolase were controlled as a group (pleitropic control) by gene expression. In *Phaseolus mungo* seedlings, enzyme assays indicated that transketolase was the rate-limiting enzyme (Ashihara and Komamine, 1974). In rat liver, some evidence was obtained that transketolase and transaldolase could be released from the large particle fraction into the cytoplasm by physiological concentrations of pentose phosphates (Baquer, *et al.*, 1972).

APPROACH II: MASS ACTION RATIOS

Mass action ratios for all the enzymes of the pathway, except isomerase and epimerase, were calculated in Krebs Ascites cells and compared with their apparent equilibrium constants at various times after the addition of glucose to the cells. Glucose-6-phosphate dehydrogenase was far removed from equilibrium at all times. Transketolase was initially close to equilibrium and then progressively departed from it (Gumaa and McLean, 1969b). In rat liver, mass action ratios indicated that 6-phosphogluconate dehydrogenase, transketolase, transaldolase, and phosphoglucoisomerase were all close to equilibrium but that glucose-6-phosphate dehydrogenase and glucokinase were removed from equilibrium (Gumaa and McLean, 1971; Greenbaum *et al.*, 1971). In *Phaseolus mungo* seedlings, again, glucose-6-phosphate dehydrogenase was identified as removed from equilibrium (Ashihara and Komamine, 1974). In HeLa cells, although mass action ratios were not calculated, it was concluded that the glucose-6-phosphate dehydrogenase reaction was not close to equilibrium and was controlled by the NADPH/NADP ratio and the concentration of metabolites (Reitzer *et al.*, 1980).

APPROACH III: CROSS-OVER PLOTS

In rat liver, although no cross-over plots were constructed, it was concluded from the levels of intermediates under various conditions that there was control at the dehydrogenases and at transketolase (Gumaa and McLean, 1968). In mouse brain, subjected to various conditions, cross-over plots indicated control at the two dehydrogenases (Kauffman *et al.*, 1969). In further experiments with rat liver, Gumaa and McLean (1971) found a clear cross-over between glucose 6-phosphate and 6-phosphogluconate, indicating control at glucose-6-phosphate dehydrogenase. In experiments with Krebs Ascites cells, Gumaa and McLean (1969b) found that, after the addition of glucose, the concentration of pentose phosphates rose linearly, while the other intermediates reached steady-state levels, and it was concluded that the pathway was operating as two independent branches synthesizing pentose phosphate, with fructose 6-phosphate appearing to be the precursor of sedoheptulose 7-phosphate.

APPROACH IV: ENZYMES UNDER HORMONAL AND ALLOSTERIC CONTROL

Starvation caused small changes in the levels of all the enzymes of the pathway in rat liver, but refeeding a high-carbohydrate diet had a marked effect on the level of glucose-6-phosphate dehydrogenase, which showed overshoot, not only being restored to normal but to supranormal levels (Novello, *et al.*, 1969). As described in Chapter 8, this increase appeared to be due to an insulin-induced synthesis of the enzyme. There was a similar result with rat adipose tissue, but it was transketolase that showed the overshoot phenomenon (Gumaa *et al.*, 1969b). As mentioned under Approach I, Eggleston and Krebs (1974)(Krebs and Eggleston, 1974) had identified a coarse control of glucose-6-phosphate dehydrogenase in rat liver by enzyme induction on feeding and a fine control by the NADPH/NADP ratio. In HeLa cells, control appeared to be by the NADPH/NADP ratio and intermediates of the pathway, but no special deinhibition by unidentified factors, as postulated by Eggleston and Krebs (1974), seemed to be necessary (Reitzer *et al.*, 1980).

In a mutant of *E. coli* possessing a glucose-6-phosphate dehydrogenase level 10 times the normal level, the flux of glucose through the oxidative branch was only 40% higher than normal, and it was concluded that hitherto unidentified metabolites were regulating the enzyme or that its kinetic properties in the cell differed from those determined *in vitro* (Orthner and Pizer, 1974).

APPROACH V: ENZYMES THAT CHANGE IN RESPONSE TO CHANGES IN THE ENVIRONMENT

In *Candida utilis* it was shown that transferring the yeast from a complex medium containing amino acids to a nitrate medium increased the specific activities of both glucose-6-phosphate dehydrogenase and transketolase by a factor of 2.6, while the other pathway enzymes were relatively unaffected (Osmond and Ap Rees, 1969). In rat skeletal muscle, regeneration of the fibers was accompanied by an approximately ninefold increase in the specific activities of the two dehydrogenases, while the four remaining enzymes of the pathway changed relatively little (Wagner *et al.*, 1978).

CONTROL OF THE OXIDATIVE BRANCH OF THE PATHWAY BY AN ACTIVATING FACTOR

It was pointed out by Sapaz-Hagar *et al.*, (1973) that there was an imbalance between the activities of 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase in rat liver in starvation and in lipogenesis and that, at physiological concentrations of NADP and NADPH, the flux through the oxidative pathway would be too low to supply significant amounts of NADPH unless some unknown activating factor was present. This point was taken up, and the control of the oxidative branch in rat liver was investigated carefully by Eggleston and Krebs (Eggleston and Krebs, 1974; Krebs and Eggleston, 1974). The NADPH/NADP ratio *in vivo* is in the range 90–250, whereas *in vitro* experiments indicated that glucose-6-phosphate dehydrogenase was completely inhibited at ratios above 9. Eggleston and Krebs concluded that *in vivo* the enzyme was somehow deinhibited, and they tested over 100 cell constituents for a deinhibitory effect. Of these, only AMP and the oxidized form of glutathione released the inhibition. The concentrations of AMP required were at the unphysiological level of 1 m*M*, whereas glutathione was effective at normal tissue concentrations (10–100 μM). Glutathione was effective on preincubation with the enzyme, and the effect was not due to reoxidation of NADPH by glutathione reductase. An unstable dialyzable cofactor was involved. Such a cofactor was subsequently isolated from mussel hepatopancreas and rat liver (Rodriguez-Segade *et al.*, 1978, 1979) and shown to be a polypeptide of molecular weight 15,000 and 10,000, respectively, and to have a differential action on the various isozymes of the two dehydrogenases (Rodriguez-Segade *et al.*, 1980).

A critical appraisal of the results of Eggleston and Krebs and of Rodriguez-Segade and co-workers has been made by Levy and Christoff (1983).

They claimed that the reversal of NADPH inhibition by glutathione was an artifact and that the glutathione effect disappeared when correct procedures were used to set up controls and inhibit glutathione reductase. No cofactor like that previously reported could be found in rat liver. It was suggested that glucose-6-phosphate dehydrogenase could be regulated by enzyme-catalyzed thiol-disulfide exchange reactions and that the factor discovered by Rodriguez-Segade's group might be an enzyme of this type.

MATHEMATICAL MODELS

A computer simulation of the pathway in lactating rat mammary gland has been constructed. However, it was impossible to construct a completely "steady" steady-state model, and this was attributed, in part, to the behavior of the NADPH/NADP couple, which first became rapidly oxidized and then slowly reduced (Haut, *et al.*, 1974). The known inhibitory effect of NADPH was not incorporated into the model, and the deinhibition described previously was only discovered later. Another model describing the interaction between the glycolysis and pentose phosphate pathways in erythrocytes showed that, in the physiological range of ATP and NADPH concentrations, the two pathways functioned almost independently. When the NADPH level fell below 80% of the physiological value the ATP level no longer remained steady, and its decrease, by its effect on phosphofructokinase, led to a proportional decrease in flux through the pentose phosphate pathway (Ataullakhanov *et al.*, 1981a).

An elaborate model of the gluconeogenic, glycolytic, and pentose phosphate pathways in isolated hepatocytes was set up by Crawford and Blum (1983). The rates of flux through the various reactions of the pathway were computed and compared with the rates obtained by the authors in the laboratory. Good agreement was obtained between the predicted and the experimental rates. Although the net flux through the pentose phosphate pathway was small, the bidirectional fluxes were large. The use of the formulation of the L-type pathway (Longenecker and Williams, 1980a) in place of the classical pathway did not provide a good fit to the experimental data.

CONCLUSION

Taking into account the results of all the various approaches, it appears that glucose-6-phosphate dehydrogenase and transketolase are the two principal enzymes controlling the two branches of the pathway, with some

indications that there may also be control at 6-phosphogluconate dehydrogenase and transaldolase. Most workers are agreed that the oxidative branch is controlled by the rate of reoxidation of NADPH, and the exact role played by the peptide factor discovered by Rodriguez-Segade and coworkers remains to be elucidated. The control of the nonoxidative branch at the transketolase step seems less well-established, particularly if one takes into account the reservations outlined earlier concerning a possible coupling with transaldolase and the manner in which transaldolase activity has been determined. To date, there have been no reports of allosteric activation or inhibition of transketolase or transaldolase, and further advances in our understanding may come from this direction.

10

Clinical and Nutritional Aspects

THE ASSAY OF BLOOD TRANSKETOLASE

In 1958, Bruns *et al.* (1958a) described an assay procedure for transketolase in blood serum and in hemolysates. The procedure involved incubation of the sample with ribose 5-phosphate and determination of the sedoheptulose 7-phosphate produced by the colorimetric cysteine–sulfuric acid reaction of Dische (1953). The true substrates of the reaction were xylulose 5-phosphate produced from ribose 5-phosphate by the action of isomerase and epimerase in the sample and ribose 5-phosphate itself. The sedoheptulose 7-phosphate that was determined represented the amount formed by transketolase during the incubation *less* any removed by the action of transaldolase or other enzymes present (e.g., aldolase, fructose-6-phosphate kinase). Values were obtained for transketolase in hemolysates, for sera from a number of species, and for human sera in various pathological states. The enzyme levels in eight different species were found to be between 24 and 96 times higher in red blood cells than in the serum. A marked elevation of the serum enzyme level was evident in uremia and virus hepatitis, and a fall in the level was evident in diabetes. The lead given by Bruns *et al.* has not

been followed, and later workers have confined themselves to measuring the much higher level of transketolase in red or white cells, rather than the low level in serum.

At around the same time, Brin *et al.* (1958) were able to show that the evolution of $^{14}CO_2$ from [2-^{14}C]glucose on its second pass through the pentose phosphate pathway reflected the activity of the transketolase–transaldolase enzyme pair. This evolution was depressed in hemolysates from animals suffering from thiamine deficiency and restored when the animals were treated with thiamine. The method, however, was cumbersome for clinical use, and in 1960 Brin *et al.* introduced an improved colorimetric procedure and showed that it could be applied to rat blood hemolysates that had been stored for some weeks in the frozen state. The procedure involved incubation of ribose 5-phosphate with a hemolysate and the formation of hexose phosphate by the operation of the pathway. The disappearance of pentose phosphate was measured colorimetrically by an orcinol reaction and the formation of hexose phosphate by an anthrone reagent. A special feature of the procedure was to measure the changes in pentose phosphate and hexose phosphate in the presence of thiamine pyrophosphate added to the incubation tubes. The percentage increase in transketolase activity was termed the "TPP effect," and it has proved to be a useful measure of the extent to which transketolase in the cells is saturated with its coenzyme and, consequently, a measure of thiamine deficiency.

In 1962, Dreyfus introduced a micromodification of the procedure of Bruns *et al.* (Dreyfus, 1962; Dreyfus and Moniz, 1962), and nearly all colorimetric procedures employed subsequently for clinical use have been based on either the procedure of Brin *et al.* (1960) or that of Dreyfus (1962). A summary of the principal procedures or modifications that have been described is given later, and the values reported for the transketolase levels and the TPP effect in normal human blood are listed in Table 10.1.

A combined enzymatic and fluorimetric method was introduced by Cheng *et al.* (1969) in which the glyceraldehyde phosphate formed from ribose 5-phosphate by transketolase was reduced to glycerophosphate in the presence of NADH, and the NAD formed was treated with alkali and measured fluorimetrically. This procedure was developed for the assay of the enzyme in liver but could possibly be adapted for use with hemolysates. Warnock (1970) introduced a modification of the Brin method in which readings were taken at 580 and 670 nm after development of the orcinol color. The object was to measure the pentose phosphate disappearing more accurately and to correct for the conversion of ribose 5-phosphate into ketopentose phosphates. The procedure also measured the rate of sedoheptulose 7-phosphate formation and corrected for variations in haematocrit of the blood samples. Hoffman *et al.* (1971) introduced a micromodification of the method of Bruns *et al.* (1958a) designed to correct for the presence of

TABLE 10.1

Transketolase Content of Normal Human Blood[a]

Mean activity (Units/l)	TPP effect (%)	Reference
78.3	—	Bruns *et al.* (1958a)
5.3 − 6.3	—	Dreyfus (1962)
62.5 ± 14.0	22.5 ± 9	Schouten *et al.* (1964)
—	20 − 14	Markkanen and Kalliomaki (1966)
125 ± 10	0	Fennelly *et al.* (1967)
56 ± 1.8	7.0 ± 0.6	Akbarian and Dreyfus (1968)
51.4 ± 2.7[b]	—	Wells *et al.* (1968)
59 ± 16.8	24.2 ± 14.9	Konttinen and Viherkoski (1968)
64.1 ± 11.0[c]	6.7 ± 5.4	Massod *et al.* (1971)
107[d]	11.0 ± 4.5	Smeets *et al.* (1971)
5.2 ± 0.4[b]	—	Lonergan *et al.* (1971)
59.7 ± 12.7	—	Wells and Marks (1972)
32	16.6	Nicholas *et al.* (1974)
35.5 ± 7.9	21.8 ± 9.0	Watson and Dako (1975)
40.6	—	Warnock (1975)
41.8 ± 1.7[b]	—	Labadarios *et al.* (1977)
69.3 ± 8.6	14.6 ± 6.4	Reijnierse *et al.* (1978)
66.5 ± 17.3	22.6	Van Zantzen *et al.* (1980)
26.5 ± 3.5	11.6 ± 11.5	Kuriyama *et al.* (1980b)
165	11.6	Delacoux *et al.* (1980)
45.3 ± 4.8	7.3 ± 5	Waldenlind *et al.* (1981)
85.5 ± 8.2	11	Waring *et al.* (1982)

[a]Values are given as the mean ± standard deviation unless otherwise indicated.

[b]Standard error of the mean.

[c]Units quoted by the authors as milliunits/1.

[d]Calculated from units/g hemoglobin, using the mean value of 13.9 g hemoglobin/100 ml blood given by Wells *et al.* (1968)

ribose 5-phosphate and glucose in the incubation mixture. Schouten *et al.* (1964) introduced a macroversion of the Dreyfus procedure, as did Massod *et al.* (1971), who replaced the inhibitory phosphate buffer with glycylglycine and corrected for haematocrit. An enzymatic procedure based on the more conventional assay for transketolase employed by enzymologists was introduced by Smeets *et al.* (1971). The glyceraldehyde phosphate formed from ribose 5-phosphate by transketolase was allowed to accumulate over a 15-min period and was then measured with glycerophosphate dehydrogenase, triose phosphate isomerase, and NADH, in the usual manner, the fall in absorbance at 340 nm being a measure of the transketolase activity. No evidence was found that triose phosphate was being lost from the

reaction mixture by the action of other enzymes, and the addition of iodoacetate to inhibit glyceraldehyde-phosphate dehydrogenase was found to be unnecessary. The activities obtained tended to be higher than with the colorimetric method, and this was attributed to the use of tris buffer instead of phosphate.

A radiochemical method claimed to be more sensitive than the colorimetric methods was introduced by Reijnierse *et al.* (1978). The method involved the conversion of [^{14}C]ribose 5-phosphate (prepared from [^{14}C]ribose and ATP) to [^{14}C]sedoheptulose 7-phosphate. The latter compound was isolated on a Dowex 1 × 8 column and was counted. The method correlated very well with results obtained using the procedure of Schouten *et al.* (1964). The authors also gave a useful review of other assay procedures and the values obtained for the TPP effect. A semiautomated method was introduced by Van Zantzen *et al.* (1980), in which the hexose phosphate formed was estimated using glucose-6-phosphate dehydrogenase in a centrifugal fast analyzer. The method correlated reasonably well with the colorimetric method of Warnock (1970) and allowed the activities of large numbers of samples to be determined. A method using the autoanalyzer has been described by Waring *et al.* (1982).

Markkanen and Peltola (1970) introduced a procedure for measuring the transketolase activity of leucocytes in the blood. They showed that the enzyme content was some 100 – 150 times higher than that in the red cell. In patients, a small fall in the mean white cell activity was detected in diabetes, chronic alcoholism, and azotemia, while a more significant fall was found in multiple myeloma (Markkanen and Peltola, 1971b).

NORMAL VALUES FOR BLOOD TRANSKETOLASE

Values for the blood transketolase levels of healthy men and women are given in Table 10.1, together with the percentage TPP effect. When values were not quoted in international units per liter in the original publication, they have been converted to these units so that comparisons may be made. The mean values for transketolase range from approximately 5 to 165 units/liter and probably reflect the different incubation times used, differences in the method of measuring concentrations of the substrate or product, and variations in the type of buffer. The values for the TPP effect lie between 0 and 24% and probably reflect the nutritional status of the normal subjects, as well as variations in the procedure.

A number of workers have reported a small difference between the sexes in the mean value of the transketolase content of red blood cells (Markkanen and Kalliomaki, 1966; Warnock, 1975; Van Zantzen *et al.* 1980), but this

difference disappears when the value is corrected for differences in erythrocyte count or hemoglobin content. A statistically significant difference between transketolase values for Caucasians and for Negroes was reported by Warnock (1975), but it was doubted whether it had any physiological importance. A small but definite decline in transketolase level with increasing age was demonstrated by Markkanen *et al.* (1969) in a group of 414 subjects, but no differences between the sexes could be detected in any age group.

THE "TPP EFFECT"

The TPP effect was introduced by Brin and his group as a useful indicator of thiamine deficiency. A value for the effect greater than 15% was taken as indicative of a thiamine deficiency (Brin, 1964; Table 10.2). Akbarian and Dreyfus (1968) recommended that a value greater than 20% should be considered as marking a deficiency, whereas, Markkanen and Kalliomaki (1966) found the effect to be very variable and of little use or reliability. However, Kuriyama *et al.* (1980b) have reported it to be one of three parameters most useful in the diagnosis of Beriberi. It has been reported that the TPP effect is pH dependent and is greater at lower pH

TABLE 10.2

The Stages of Thiamine Deficiency[a]

Stage	Day of onset	Symptoms and comments
Preliminary	5	Inadequate dietary thiamine, malabsorption, abnormal metabolism, urinary thiamine reduced to 50 μg/day
Biochemical	10	Blood transketolase level depressed, TPP effect > 15%, urinary thiamine 25 μg/day
Physiological	21–28	Loss of appetite, malaise, insomnia, irritability, blood transketolase 75–85% normal, TPP effect > 30%, urinary thiamine 0–25 μg/day
Clinical	30–300	Polyneuritis, intermittent claudication, bradycardia, peripheral oedema, cardiac enlargement, opthalmoplegia, blood transketolase < 65% normal, TPP effect > 40%, urinary thiamine negligible
Anatomical	200 plus	Cardiac hypertrophy, degeneration of granular layer of cerebellum, cerebral hemorrhage, degeneration of neurons and processes, blood transketolase < 55% normal, TPP effect approximately 50%

[a]From Brin (1964). *J. Am. Med. Assoc.* 187, 762–766. Copyright 1964, American Medical Association.

values, which is in accord with the tighter binding of the cofactor to the Baker's yeast enzyme that was noted as the pH decreases (see p. 81). The importance of adequate buffering when the TPP effect is measured was emphasized (Tate *et al.* 1984).

From his experiments with both rats and humans, Dreyfus (1962) observed that the TPP effect increased as the deficiency of thiamine became more intense. However, it never approached 100%, and it was concluded that, when there was a high degree of deficiency, the amount of apotransketolase available to bind added coenzyme also fell.

MEASUREMENTS OF OTHER ENZYMES

Bruns (1956) described a procedure for the assay of isomerase in blood serum. Of the 11 species examined, man had the lowest content and rabbit the highest. The mean serum value for unhemolyzed blood from 21 individuals was 3.5 μmol/hr/ml. The value was raised in a number of diseases of the lymph glands and in chronic nephritis. The isomerase content of the red blood cell was about 1000 times that of the serum, and measurements were later extended to the whole blood of the 11 species and to the various tissues of the mouse (Bruns *et al.* 1958b).

CLINICAL STUDIES OF VARIOUS PATHOLOGICAL AND PHYSIOLOGICAL STATES

A summary of the changes in blood transketolase levels and the TPP effect found by various workers is given in Table 10.3. It soon became clear from the results of Brin and his group on rats (Brin *et al.* 1960; Brin, 1962) and of Dreyfus (1962) on patients that thiamine deficiency was reflected in a fall in transketolase activity in blood and other tissues. Gradually, as a result of the efforts of Brin, measurements of blood transketolase levels and of the TPP effect have come to be accepted as an important means of diagnosing this deficiency, which is discussed in detail later. The measurements of Markkanen and co-workers on large numbers of patients have revealed changes in transketolase levels in a number of other conditions and are described in the next section, together with the contributions from other groups.

Transketolase and Thiamine Deficiency in Man

This topic has been widely reviewed (Brin, 1962, 1964, 1966, 1967a,b, 1970; Dreyfus, 1962, 1967; Embree and Dreyfus, 1963). Brin showed that in

TABLE 10.3

Blood Transketolase Levels in Various Physiological and Pathological States

State	TK level[a]	TPP effect[a]	Reference
Man			
Beriberi heart disease	D	U	Akbarian and Dreyfus (1968)
Beriberi	D	U	Kuriyama *et al.* (1980b)
Polyneuritis	D	N	Kjosen and Seim (1977)
Partial gastrectomy	D	N	Markkanen and Kalliomaki (1966)
Encephelopathy	D	U	Delacoux *et al.* (1980)
Alcoholic cirrhosis	D	N	Fennelly *et al.* (1967)
Chronic alcoholism	D	—	Markkanen and Peltola (1971a)
	D	U	McLaren *et al.* (1981)
Cirrhosis	D	U	Labadarios *et al.* (1977)
Alcoholism	D	U	Waldenlind *et al.* (1981)
Hepatic necrosis	D	U	Labadarios *et al.* (1977)
Senile dementia	D	—	Markkanen and Peltola (1971a)
Fasting	D	U	Haro *et al.* (1966)
Pernicious anemia	U	N	Kjosen and Seim (1977)
Addison pernicious anemia	U	N	Markkanen and Kalliomaki (1966)
	U	—	Markkanen (1968)
B_{12} Deficiency anemia	U	—	Wells *et al.* (1968), Wells and Marks (1972)
Folate deficiency anemia	N	—	Wells and Marks (1972)
Uremia	D	—	Lonergan *et al.* (1970)
	D	—	Lonergan *et al.* (1971)
	N	—	Markkanen *et al.* (1972)
	N	—	Warnock *et al.* (1974)
	D	D	Kuriyama *et al.* (1980a)
Hypo- and hyperthyroidism	N	—	Markkanen and Peltola (1971a)
Thyrotoxicosis	D	D	Konttinen and Viherkoski (1968)
Diabetes	D	N	Kjosen and Seim (1977)
	N	D	Watson and Dako (1975)
	N	D	Markkanen and Kalliomaki (1966)
Treated diabetes	D	—	Markkanen and Peltola (1971a)
Hepatic porphyria	D	—	Markkanen *et al.* (1971)
Sideropenia	N	—	Markkanen and Peltola (1971a)
Collagen diseases	N	D	Markkanen and Kalliomaki (1966)
Increasing age	SD	—	Markkanen *et al.* (1969)
Rat			
Thiamin deficiency plus			
Hepatic necrosis	D	—	Fennelly *et al.* (1964)
Magnesium deficiency	D	—	Zieve *et al.* (1968)
Hyperthyroidism	N	—	Appledorf and Tannenbaum (1970)

[a]D, Down; U, up; SD, slow decline; N, normal.

experimental thiamine deficiency the body weight falls, the excretion of thiamine in the urine falls to a deficient level, and the TPP effect increases. He described the successive stages of thiamine deficiency as set out in Table 10.2, and he pointed out that the biochemical deficiency may be detected from blood transketolase measurements before clinical signs of deficiency become apparent (Brin, 1964). Warnock *et al.* (1978) have shown that the blood TPP level falls some days before the transketolase level and suggested it to be a more sensitive indicator of deficiency than the latter.

Thiamine deficiency may be the result of malnutrition or fasting (Haro *et al.*, 1966), or a secondary consequence of a partial gastrectomy (Markkanen and Kalliomaki, 1966), cirrhosis or hepatic necrosis (Labadarios *et al.*, 1977), senility (Markkanen and Peltola, 1971a), and chronic alcoholism. In the latter case, persistence of the deficiency may lead to the state known as the Wernicke–Korsakoff syndrome. Symptoms of the deficiency may manifest themselves as Beriberi (Kuriyama, *et al.*, 1980b), heart disease (Akbarian and Dreyfus, 1968), polyneuritis (Kjosen and Seim, 1977), or encephalopathy (Delacoux, *et al.*, 1980). All of these diseases have been placed together in Table 10.3.

In a survey of the status of thiamine nutrition in Indians, Bamji (1970) could find no correlation between blood transketolase levels and clinical symptoms or with the urinary excretion of thiamine in response to a 1-mg load. By using the blood transketolase as a measure of adequate thiamine nutrition, it was possible to determine the minimal thiamine requirement as 0.25 mg/1000 cal for women and 0.35 mg/1000 cal for men. In a similar survey in Japan, Kuriyama *et al.* (1980b) found that people suffering from Beriberi had lowered blood transketolase and thiamine levels and an increased TPP effect. The level of blood pyruvate, on the other hand, did not show any significant difference from the normal group, and they concluded that, even using the first three parameters together, it was still not possible to make a completely accurate diagnosis of the deficiency state from laboratory measurements alone.

Measurements on normal controls and on a series of patients were carried out by Kjosen and Seim (1977). They could find no differences in blood transketolase or the TPP effect between a control group and a "malnourished" group. Patients with polyneuritis and diabetes mellitus had lower blood transketolase levels than the controls, and those with pernicious anemia had higher values. There were no significant differences in the TPP effect between patients and controls, and it was concluded that the differences found reflected variations in the levels of apotransketolase rather than in thiamine status.

A number of workers have studied the ability of thiamine administration to patients to raise the blood transketolase level and return the TPP effect to

a value within the normal range. In a study of malnourished alcoholics with cirrhosis, it was found that thiamine therapy may be useful in restoring these quantities to normal and improving neurological signs. The therapy had to be prolonged over a period of 3–6 months, and in some patients it was ineffective. The problem in these patients was believed to be an inability to convert free thiamine to a metabolically active form (Fennelly *et al.*, 1967). In a similar study of patients with alcoholic and nonalcoholic cirrhosis and with acute hepatocellular necrosis, a good proportion of the patients responded to thiamine administered intravenously for 7 days (Labadarious *et al.*, 1977). In a careful study of alcoholic patients, Waldenlind *et al.* (1981) were able to observe a return to normal of the TPP effect after oral administration of thiamine for 10 days. The blood transketolase levels were not returned to normal, however, and it was suggested that only after more prolonged therapy might this occur. In another study, thiamine was given intramuscularly to chronic alcoholics. Although the blood transketolase and thiamine pyrophosphate levels rose after treatment and the TPP effect decreased, the magnitudes of these changes were not statistically significant (McLaren *et al.*, 1981). Blass and Gibson (1977) measured transketolase activity in cultured fibroblasts from patients with the Wernicke–Korsakoff syndrome and from normal controls. They found the enzyme from patients was less able to bind thiamine pyrophosphate than the enzyme from control fibroblasts. They suggested that the patients had an inherited predisposition to develop the syndrome when exposed to a thiamine deficiency.

Transketolase and Other Pathological Conditions

Blood transketolase levels in various pathological conditions are summarized in Table 10.3. It is noteworthy that the only condition in which the blood transketolase is elevated is pernicious anemia, probably due to a higher proportion of the more transketolase-active white cells in the blood (Wells and Marks, 1972). The administration of vitamin B_{12} gradually returns the enzyme levels to normal (Wells *et al.*, 1968; Markkanen, 1968).

Conflicting reports have appeared on changes in blood transketolase levels in uremia, but a published report (Kuriyama *et al.*, 1980a) confirms previous conclusions that the blood transketolase level was reduced in this condition. The lower activity was not due to a deficiency of thiamine but to the presence of an inhibitory compound, of molecular weight less than 500, present in uremic blood before dialysis and removed by the dialysis treatment (Lonergan *et al.*, 1970, 1971; Kuriyama *et al.*, 1980a; Sterzel *et al.*, 1971). Phosphate ion could be the substance responsible, but this possibility remains to be explored. It has been suggested that a low level of transketolase activity was connected with the development of uremic neuropathy

(Lonergan *et al.*, 1970), but no evidence for this hypothesis could be found by Warnock's group (Warnock *et al.*, 1974).

Glucose-6-Phosphate Dehydrogenase Deficiency and Hemolytic Anemia

The subject of glucose-6-phosphate dehydrogenase deficiency and hemolytic anemia has been well reviewed by Marks (1964) and by Yoshida (1973). The latter has pointed out that the correlation between the degree of enzyme deficiency, the properties of glucose-6-phosphate dehydrogenase in deficient subjects, and the severity of clinical symptoms was very poor, and he suggested that it would be necessary to investigate the response of the variant enzyme to simulated physiological conditions before the clinical symptoms could be satisfactorily explained. Some of his interpretations have been challenged by Kirkmann and Gaetani (1975).

It has been shown that the small amount of dehydrogenase in the erythrocytes of deficient individuals was already operating at a rate close to maximal and was unable to provide more NADPH when a small oxidative stress was applied to the cells (Brand, *et al.*, 1970b; Gaetani *et al.*, 1974). As a result, the level of reduced glutathione fell, the structure of the cell membrane was disrupted, and hemolysis ensued. When cells were incubated with α-naphthol, the level of reduced glutathione was lowered. In normal cells, even when incubated with α-naphthol the NADPH/(NADP + NADPH) ratio remained at 0.9. In deficient cells, however, this ratio fell from 0.19 to 0.07 when so treated. These results supported the theory that a low concentration of NADPH is insufficient to keep glutathione reduced and that the cells hemolyze under an oxidative stress (Mareni and Gaetani, 1976).

Children with a glucose-6-phosphate dehydrogenase deficiency suffer severe hemolytic consequences if they swallow mothballs containing naphthalene, which is subsequently metabolized to naphthol (Gaetani *et al.*, 1974). Later, it was shown that in deficient erythrocytes the dehydrogenase is under some restraint so that the NADPH/NADP ratio is only 0.33. When the cells are hemolyzed this restraint is released, and the ratio increases to 0.89 in the hemolysate. In normal cells the ratio is 0.98 in the intact cell, increasing to 0.99 on hemolysis (Galiano *et al.*, 1978).

When normal erythrocytes were stressed by exposure to peroxide and treatment with sodium azide, the activity of the oxidative branch increased some 15-fold, but no increase was observed with dehydrogenase-deficient cells. Although peroxidation of lipids and a fall in arachidonic acid levels were observed in both types of cell, there was no significant difference between them and there was no difference in the degree of hemolysis

induced. It was concluded that oxidative stress alone could not account for the hemolysis of deficient cells and that other factors, possibly involving the whole organism, played a part (Benatti *et al.*, 1981). However, it has been shown that the lipids of deficient cells are more oxidized and more fluid and contain polypeptide aggregates that can be dissociated by dithiothreitol but which are absent from normal cells (Rice-Evans *et al.*, 1981).

In subjects suffering from dehydrogenase deficiency of the fava anemia type, the levels of transketolase and transaldolase had increased to almost double the normal levels, possibly as some sort of compensation mechanism (Bonsignore *et al.*, 1961). Antimalarial drugs, such as paludrine, daraprim, chloroquine, mepacrine, and primaquine, were able to inhibit the dehydrogenases of yeast, skin, and erythrocytes to a marked degree, and it was suggested that this was the cause of the hemolytic and cutaneous side effects of antimalarial therapy with these drugs (Cotton and Sutorius, 1971).

Xylitol Metabolism

Xylitol has been used in a number of countries as a parenteral nutrient (for a review see Horecker *et al.*, 1969). Adverse reactions have been reported to include deposits of calcium oxalate crystals in the intracranial arteries and kidneys. Consequently, the metabolism of xylitol has become a subject of interest to clinicians. Xylitol is first oxidized to D-xylulose, which is then phosphorylated to D-xylulose 5-phosphate and further metabolized via the pentose phosphate pathway. Patients infused with xylitol excrete considerable amounts of glycolic and threonic acids. The glycolic acid is suggested to arise from further oxidation of glycolaldehyde released from its transketolase-bound complex with thiamine pyrophosphate, and threonic acid, by the further metabolism of erythrose 4-phosphate (Chalmers *et al.*, 1975).

On the other hand, Barngrover *et al.* (1981) have proposed that D-xylulose is converted to D-xylulose 1-phosphate by the liver fructokinase and then split into dihydroxyacetone phosphate and glycolaldehyde by aldolase. The glycolaldehyde is oxidized to glycolate, converted to glyoxylate, and further oxidized to oxalate. The metabolism of xylitol has also been investigated by Quadflieg and Brand (1978).

Diabetes

Sochor *et al.* (1979a,b) have produced evidence that in the diabetic kidney, enzyme levels of the pentose phosphate and glucuronate-xylulose pathways are raised, in line with the idea that, in diabetes, non-insulin-requiring tissues tend to overutilize glucose.

Other Conditions

The cardioprotective action of ribose and its ability to maintain adenine nucleotide levels has aroused considerable interest. It has been suggested that in the myocardium the activity of the pentose phosphate pathway is low and inadequate and that ribose when administered bypasses the pathway and stimulates nucleotide synthesis by increasing the size of the phosphoribosyl pyrophosphate pool (Zimmer *et al.*, 1984). In sickle-cell disease it appeared that the functioning of the pathway was impaired, resulting in a decrease in the level of reduced glutathione and an increase in hemolysis and Heinz body formation (Lachant *et al.*, 1983).

STUDIES OF TRANSKETOLASE AND THIAMINE DEFICIENCY IN OTHER SPECIES

Erythrocytes from rats on a thiamine-deficient diet had a reduced ability to oxidize glucose 6-phosphate and accumulated pentoses (Brin *et al.*, 1958). The blood transketolase levels were depressed but could be raised by the addition of thiamine pyrophosphate *in vitro* or the administration of thiamine *in vivo* (Brin *et al.*, 1960). Similar findings were reported by Dreyfus (1962) and Dreyfus and Moniz (1962).

In further experiments, Brin (1962) showed that, in thiamine-deficient rats, the transketolase activities were decreased in all tissues examined except brain. During the 13 days of the experiment, neither growth nor behavior were affected, although transketolase levels fell, and it was concluded that the heart was the most sensitive tissue and the brain the least sensitive to thiamine deficiency. Dreyfus and Moniz (1962) demonstrated that in the central nervous system of the rat, white matter had a higher transketolase activity than gray matter. From a quantitative histochemical estimation of the enzyme, it was concluded that the activity was highest in myelinated tracts and that transketolase was involved in the development and maintenance of the myelin sheath (Dreyfus, 1965). Such an involvement would account for the neurological symptoms encountered in thiamine deficiency (Embree and Dreyfus, 1963) and in uremia (Lonergan *et al.*, 1970). It was also shown that, in rats, deficiency affects transketolase before it affects pyruvate dehydrogenase, and the suggestion was made that the susceptibility of transketolase in the heart may account for the development of heart disease in cases of human Beriberi (Dreyfus and Hauser, 1965).

In experiments with pigeons fed a normal, deficient, and thiamine-supplemented diet, the transketolase of erythrocytes, muscle, and heart showed the greatest response to changes in dietary thiamine, while brain

showed a smaller response and liver showed the least affect of all (Ostrovskii, 1963). In an attempt to cast some light on the similar condition in humans, liver necrosis was induced in rats with carbon tetrachloride. It became clear that the presence of necrosis interfered markedly with the restoration of transketolase levels by the administration of thiamine (Fennelly *et al.*, 1964). Schenker *et al.* (1971) have also confirmed previous observations (Brin, 1962; Fennelly *et al.*, 1964) that, in thiamine-deficient rats, liver transketolase falls and the TPP effect is increased. Zieve *et al.* (1968) investigated the effects of magnesium deficiency on blood and liver transketolase in thiamine-deficient rats given thiamine. Their results indicated that after 2 months of magnesium deficiency, the liver transketolase was lowered and did not readily respond to restoration of magnesium over a 2-week period.

Using [^{35}S] thiamine and mice, as experimental animals, Ostrovskii and Gorenshtein (1967) showed that transketolase was only affected when tissue stores of the coenzyme had fallen to a very low level. Thereafter, the transketolase activity became proportional to the thiamine content of the tissue. Mice on a thiamine-deficient diet were injected with 2 μg/day of [^{14}C] thiamine. There was a small but statistically significant fall in transketolase levels in the liver, kidneys, heart, and spleen and no changes in blood, skeletal muscles, stomach, and pancreas. The pyruvate dehydrogenase and oxoglutarate dehydrogenase levels in the liver and kidneys fell in a similar manner (Trebukhina *et al.*, 1983). The activity of liver transketolase in rats was shown to increase five-fold as the thiamine content of the diet was raised from 1 mg/kg to 4 mg/kg (Cheng *et al.*, 1969).

In experiments on the livers of thiamine-deficient rats, McCandless *et al.* (1975) measured the intermediates and the flux through the pathway. They reported that, apart from glucose 6-phosphate, neither the levels of the intermediates nor the flux were affected by the deficiency. As liver transketolase is known to be greatly reduced in such animals, they concluded that the pathway had a considerable reserve capacity. This conclusion, however, may not be valid as the fall in transketolase was assumed rather than demonstrated by actual measurements.

A number of workers have used thiamine antagonists to induce symptoms of deficiency. Brin (1962) injected oxythiamine and observed a fall in transketolase levels in all the nine tissues examined in both normal and deficient rats. Trebukhina and Ostrovskii (1964) injected oxythiamine into pigeons and found a fall in transketolase activities in heart and blood, no change in muscle or brain, and a marked increase in liver. This latter result contrasts with the fall in liver enzyme activity found by other workers, using the rat (Brin, 1962; Benevenga *et al.*, 1964). Benevenga *et al.* (1964) added oxythiamine to the thiamine-free diet of rats and obtained a fall in liver

transketolase additional to that produced by the vitamin deficiency. Deficient mice treated with pyrithiamine showed a 50% fall in both brain and liver transketolase (Holowach *et al.*, 1968).

THE MECHANISM OF ACTIVATION OF TRANSKETOLASE BY THIAMINE

Working with thiamine-deficient cultures of *Lactobacillus viridescens*, Böhm *et al.* (1973) showed that the transketolase activity could be increased 16-fold by a short incubation with thiamine or 23-fold if thiamine pyrophosphate was used. Inhibitors of protein synthesis did not interfere with the activation process but disruption of the cells did. Reactivation could be obtained *in vitro* provided the cells were disrupted in the presence of thiamine pyrophosphate, magnesium, cysteine, and NADPH. It was suggested that in the disrupted cells the apoenzyme was in an unstable membrane-bound form and that thiol groups were required to preserve its structure. The results also indicated that more apotransketolase was present in deficient cultures than in those containing adequate thiamine. These observations might explain the unexpected finding by Trebukhina and Ostrovskii (1964) of a higher level of the enzyme in the liver of oxythiamine-treated rats than in the controls.

From their experiments with thiamine-deficient rat liver, Vinogradov *et al.* (1979) concluded that the cytoplasmic fraction did not bind labeled thiamine pyrophosphate and the activation of transketolase observed on administration of thiamine *in vivo* occurred at the stage of ribosomal translation.

11

Glutathione Metabolism and the Pentose Phosphate Pathway

Glutathione has a number of different functions that are not all well understood. It is involved in the reduction of key compounds in the cell and the protection of cell membranes against oxidative damage, in transport processes and the operation of the γ-glutamyl cycle, as a coenzyme, in detoxification, and as a substrate for glutathione S-transferases (Meister and Tate, 1976; Wellner and Meister, 1981). More specifically, it plays an important role in the prevention of damage by peroxides, superoxide, and hydroxyl radicals produced within the cell.

Hydrogen peroxide in low concentrations was removed by glutathione peroxidase more efficiently than by catalase (Mills, 1957), and reduced glutathione was regenerated by an NADPH-dependent glutathione reductase. In some tissues, and in erythrocytes in particular, the main source of NADPH is the oxidative branch of the pentose phosphate pathway, so the pathway is linked to hydrogen peroxide removal, as shown in Fig. 11.1 (Cohen and Hochstein, 1963).

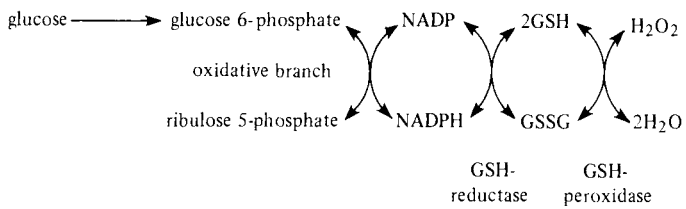


Fig. 11.1. Coupling of hydrogen peroxide removal to the oxidative branch of the pentose phosphate pathway.

Further evidence for this sequence of events in red blood cells was provided by Jacob and Jandl (1966), who showed that the rate of $^{14}CO_2$ production from [1-^{14}C]glucose was regulated by the ratio of oxidized glutathione to reduced glutathione. Increasing the ratio by the addition of peroxide or a partial blockage of glutathione reduction by the addition of *N*-ethylmaleimide increased the rate of the oxidative branch. Complete blockage of glutathione reduction by *N*-ethylmaleimide depressed the rate almost completely, and sustained low levels of hydrogen peroxide stimulated the pathway. A similar linkage between glutathione reduction and the oxidative branch was shown in rat liver (Hochstein and Utley, 1968). The stimulation of the oxidative branch by oxidized glutathione may take place either by an increase in the rate of NADP turnover or by a direct action on glucose-6-phosphate dehydrogenase (Eggleston and Krebs, 1974; see Chapter 9).

In mouse brain, changes in the level of reduced glutathione brought about by the administration of drugs had a parallel effect on the oxidative branch of the shunt, and from these and previous experiments, it was concluded that the latter played a major role in brain in maintaining glutathione and the cellular milieu in the reduced state (Hotta, 1962; Hotta and Seventko, 1968). In Ehrlich Ascites cells, glutathione peroxidase and reductase were coupled together, and the oxidative branch of the pathway was controlled by the level of reduced glutathione. Glutathione reductase was purified from the cells and shown to be first stimulated and then inhibited as the concentration of *N*-ethylmaleimide was gradually increased. In the intact cells there was a similar effect of the inhibitor on the evolution of labeled carbon dioxide from [1-^{14}C]glucose, showing the close relationship between glutathione reductase and shunt activity (Hosoda and Nakamura, 1970).

May (1981) produced evidence to support the concept that in rat adipocytes changes in the GSH/GSSG ratio, either directly or indirectly via changes in the NADPH/NADP ratio, controlled the pentose phosphate pathway, which, in turn, provided reducing equivalents for the reduction of glutathione. In leucocytes, Reed (1969) showed that glutathione oxidation by hydrogen peroxide increased the activity of the oxidative branch. In

platelets, shunt activity was increased when *t*-butyl peroxide or arachidonic acid was added, and it was suggested that glutathione peroxidase acted to reduce hydroperoxyeicosatetraenoic acids (HPETE), the primary product of arachidonic acid oxidation by lipoxygenase, to hydroxyeicosatetraenoic acids (HETE) (Fig. 11.2; Bryant *et al.*, 1982).

By using a carbon dioxide electrode to measure carbon dioxide production in a suspension of erythrocytes, the rate of the oxidative branch could be measured directly, and the rate varied by adding *t*-butyl hydroperoxide as a substrate for glutathione peroxidase. It was shown that the rate of carbon dioxide formation was proportional to the rate of delivery of peroxide up to a certain maximum. When the rate of the oxidative branch was varied from 0 to 60% of the rate corresponding to this maximum, the concentration of reduced glutathione remained practically unchanged at a value greater than 90% of the total glutathione, but when the rate was further increased the reduced glutathione concentration was no longer maintained and fell to zero (Ataullakhanov *et al.*, 1981b). Perfusion of the livers from fed and from fasted rats with *t*-butyl hydroperoxide led to a fall in the GSH/GSSG ratio. In fasted rats, glucose-6-phosphate dehydrogenase levels and the rate of the oxidative branch of the pathway were increased, but in the fed animals there was no change in the rate of the oxidative branch (Brigelius, 1983).

The activity of glutathione peroxidase in mouse lung was reduced, without altering the activities of glutathione reductase and the two oxidative branch dehydrogenases, by giving the animal a selenium-free diet. Normally, when an animal is exposed to the oxidative stress of ozone in the inspired air, the activity of the shunt is stimulated. However, in selenium-deficient animals this stimulation did not take place, again indicating the relationships between shunt activity, glutathione reduction, and protection against oxidation (Elsayed *et al.*, 1982).

The parasitic amoeba *Entamoeba histolytica* does not contain glutathione, glutathione reductase, glutathione peroxidase, or γ-glutamyl transferase

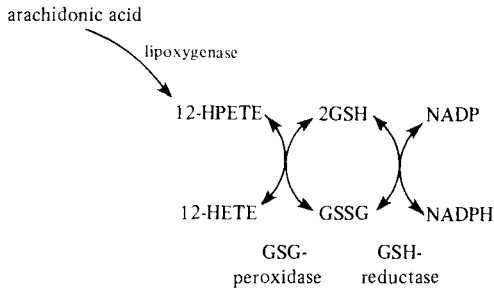


Fig. 11.2. Coupling of arachidonic acid oxidation and glutathione turnover.

and is apparently a eukaryote without glutathione metabolism. It was proposed that protection against oxygen toxicity is one of the primary functions of glutathione and that in this organism, lacking mitochondria and the usual aerobic respiratory pathways, such protection is unnecessary (Fahey *et al.*, 1984). In this context it is of interest to note that the oxidative branch of the pentose phosphate pathway, which normally helps to keep glutathione reduced, is also missing (see p. 101).

In erythrocytes, an inability of the cell to maintain high levels of NADPH may lead to a lowering of cellular concentrations of reduced glutathione and a lowered resistance to oxidative stress. The clinical consequences of this in glucose-6-phosphate dehydrogenase deficiency are discussed in Chapter 10.

12

Use of Radioisotopes in Elucidating the Nature of and Quantitating the Pentose Pathway [1]

INTRODUCTION

Professor Terry Wood in Chapter 2 concludes that one might regard the studies with radioisotopes as having created more problems than they have solved. He further states that in his opinion to unequivocally establish the nature of the pathway (and hence allow its quantitation), radioactive isotopic studies would best be eschewed and reliance placed on unambiguous chemical methods. My associates and I have a different view, and he has

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graciously asked me to prepare this chapter detailing the basis for that view.

An analogy may help. In the United States there are two pathways generally considered for automobile travel between New York and Chicago. One pathway travels through the cities of Philadephia and Pittsburgh via the New Jersey and Pennsylvania highways (turnpikes). Another pathway travels through the cities of Buffalo and Cleveland using the New York highway (thruway). Let us assume you knew cars traveled by one or the other pathway. How could you tell which pathway had been used? One way would be to examine the intermediate cities, i.e., Philadelphia, Buffalo, etc. One might find that on examination one or the other would be favored, for example, by condition of bridges, repair of roads within the city, etc., (specificity, capacity). However, since isolating the city is required to make this examination, the findings could be misleading (effect of surrounding environment, annual snowfall, concentration of local cars that might interfere with the flow of traffic being lost in isolation, etc.).

On the other hand, suppose from the description of the highways it must be that if six cars departed New York and in the order 1, 2, 3, etc., they would arrive in a different order in Chicago if they traveled via the New Jersey and Pennsylvania highways, say the order 6, 5, etc., then if they went via the New York highway, say the order 2, 3, etc. Then, if you could in some manner, without perturbing the system, determine the order in which they arrive (tracing with label comes to mind), you could establish which pathway was followed.

Of course, suppose there was another pathway between New York and Chicago, say via Cincinnati, Ohio, by which the same order of arrival in Chicago as you observed would occur. You could not eliminate that other pathway. However, your tracing would eliminate the pathway giving the order you did not observe. As a result, the tracing could not unequivocally establish the intermediates in the pathway. This might well have to be based on the characteristics of the cities (albeit circumstantial evidence).

Most early attempts to estimate the extent of participation of the pentose cycle relative to the Embden–Meyerhof pathway in the metabolism of glucose by various tissues were based on premises that proved untenable, and these are only of historical interest. They are discussed in reviews by Wood (1955) and Katz (1961). Methods presently available and their theoretical bases have also been reviewed (Wood *et al.*, 1963; Landau and Katz, 1965; Flatt and Ball, 1965). Refinements of the methods are to be found in Katz *et al.* (1966), Landau and Bartsch (1966), and Katz and Rognstad (1967). Estimates are made from determinations of the fate of the labels of specifically labeled glucoses in their metabolism to various metabolic products.

THE NATURE OF THE PATHWAY

The "Classical" Pathway

Studies, primarily by Warburg, Lipmann, Dickens, and Horecker defined the oxidative portion of the pentose pathway. C-1 of glucose 6-phosphate is oxidized to CO_2, and pentose 5-phosphate is formed, i.e., glucose 6-phosphate → 6-phosphogluconate → CO_2 + pentose 5-phosphate. In most circumstances in which the oxidative portion operates, the need for NADPH should exceed the need for pentose 5-phosphate. Therefore, in the formation of the NADPH, to avoid accumulation, the pentose 5-phosphate has to be metabolized further. An early indication that the pathway was cyclic was the demonstration that degradation of pentose nucleosides by red cells was accompanied by hexose phosphate and triose phosphate formation (Dische, 1938). Subsequently, glucose 6-phosphate was found to be a product of ribose 5-phosphate metabolism by liver, bone marrow, and spinach leaf preparations.

Studies followed defining the activities of transaldolase and transketolase and demonstrating that in the conversion by a liver enzyme preparation of pentose 5-phosphate to hexose 6-phosphate, sedoheptulose 7-phosphate appeared to be an intermediate. This led to the postulate (Horecker *et al.*, 1954; Horecker and Mehler, 1955) of the sequence of reactions that now comprise the nonoxidative portion of the classical pathway (see Chapter 2, Fig. 2.1):

$$\text{xylulose 5-phosphate} + \text{ribose 5-phosphate} \xrightarrow{\text{transketolase}} \text{glyceraldehyde 3-phosphate} + \text{sedoheptulose 7-phosphate}$$

$$\text{sedoheptulose 7-phosphate} + \text{glyceraldehyde 3-phosphate} \xrightarrow{\text{transaldolase}} \text{erythrose 4-phosphate} + \text{fructose 6-phosphate}$$

$$\text{xylulose 5-phosphate} + \text{erythrose 4-phosphate} \xrightarrow{\text{transketolase}} \text{glyceraldehyde 3-phosphate} + \text{fructose 6-phosphate}$$

Thus, in sum: 3 pentose 5-phosphate → 2 fructose 6-phosphate + glyceraldehyde 3-phosphate. Since phosphohexose isomerase is a relatively active enzyme in tissues where it is present, the pathway is a cycle (Wood *et al.*, 1963) in which 3 glucose 6-phosphate → 3 CO_2 + glyceraldehyde 3-phosphate + 2 glucose 6-phosphate.[2]

[2] If six molecules of glucose 6-phosphate are metabolized in the pathway and two molecules of the glyceraldehyde 3-phosphate formed are converted, to glucose 6-phosphate, for example, in liver, the overall reaction is 6 glucose 6-phosphate → 6 CO_2 + 5 glucose 6-phosphate (see Chapter 2, Fig. 2.2).

$$\underset{\text{Xu-5-P}}{2\,3\,4\,5\,6} + \underset{\text{R-5-P}}{2\,3\,4\,5\,6} \underset{\leftarrow}{\overset{\text{TK}}{\rightleftharpoons}} \underset{\text{S-7-P}}{2\,3\,2\,3\,4\,5\,6} + \underset{\text{GAP}}{4\,5\,6}$$

$$\underset{\text{S-7-P}}{2\,3\,2\,3\,4\,5\,6} + \underset{\text{GAP}}{4\,5\,6} \underset{\leftarrow}{\overset{\text{TA}}{\rightleftharpoons}} \underset{\text{F-6-P}}{2\,3\,2\,4\,5\,6} + \underset{\text{Er-4-P}}{3\,4\,5\,6}$$

$$\underset{\text{Xu-5-P}}{2\,3\,4\,5\,6} + \underset{\text{Er-4-P}}{3\,4\,5\,6} \underset{\leftarrow}{\overset{\text{TK}}{\rightleftharpoons}} \underset{\text{F-6-P}}{2\,3\,3\,4\,5\,6} + \underset{\text{GAP}}{4\,5\,6}$$

Scheme 1.

Designating the carbonyl carbon of glucose by 1 and succeeding carbons in sequence by 2–6, the movement of carbons in the nonoxidative portion of the pathway is shown in Scheme 1. Thus, the two molecules of hexose 6-phosphate formed have the distributions 232456 and 233456. Therefore, the metabolism of [2-^{14}C]glucose through one turn of the cycle, yields glucose 6-phosphate with twice as much ^{14}C in its C-1 as in C-3.

Horecker *et al.* (1954) incubated [1-^{14}C]ribose 5-phosphate, which would be formed in the oxidative pathway from [2-^{14}C]glucose, with a liver enzyme preparation for 17 hr. They isolated and degraded the glucose from the glucose 6-phosphate that was formed. The percentage distribution in the glucose's six carbons was C-1 = 74, C-2 = 0, C-3 = 24, C-4 = 1, C-5 = 0, and C-6 = 1. Thus, instead of the predicted 66% of the ^{14}C in glucose 6-phosphate in its C-1 and 33% in its C-3 (Scheme 2), there was 74% in C-1 and 24% in C-3. They also incubated [2,3-^{14}C]pentose 5-phosphate. C-2 and C-3 of pentose 5-phosphate are derived from C-3 and C-4 of glucose 6-phosphate via the oxidative portion of the pathway. Glucose 6-phosphate formed from [2,3-^{14}C]pentose 5-phosphate via the nonoxidative portion of the classical pathway should then have ^{14}C in its C-2, C-3, and C-4, with the same amount of ^{14}C in C-2 and C-4 and half that amount in C-3 (Scheme 2). The percentages found were C-1 = 7, C-2 = 28, C-3 = 20, C-4 = 45, C-5 = 1, and C-6 = 0.

These distributions were concluded by Horecker *et al.* (1954) to be in fair agreement with the sequence of reactions they postulated. This seems so considering that the [2,3-^{14}C]pentose 5-phosphate was prepared from

$$\underset{\text{Xu-5-P}}{1\,2\,3\,4\,5} + \underset{\text{R-5-P}}{1\,2\,3\,4\,5} \underset{\leftarrow}{\overset{\text{TK}}{\rightleftharpoons}} \underset{\text{S-7-P}}{1\,2\,1\,2\,3\,4\,5} + \underset{\text{GAP}}{3\,4\,5}$$

$$\underset{\text{S-7-P}}{1\,2\,1\,2\,3\,4\,5} + \underset{\text{GAP}}{3\,4\,5} \underset{\leftarrow}{\overset{\text{TA}}{\rightleftharpoons}} \underset{\text{F-6-P}}{1\,2\,1\,3\,4\,5} + \underset{\text{Er-4-P}}{2\,3\,4\,5}$$

$$\underset{\text{Xu-5-P}}{1\,2\,3\,4\,5} + \underset{\text{Er-4-P}}{2\,3\,4\,5} \underset{\leftarrow}{\overset{\text{TK}}{\rightleftharpoons}} \underset{\text{F-6-P}}{1\,2\,2\,3\,4\,5} + \underset{\text{GAP}}{3\,4\,5}$$

Scheme 2.

[3,4-^{14}C]glycogen isolated from rat liver after administering $NaH^{14}CO_3$ and ^{14}C would then be expected to have been incorporated in small amounts into carbons other than C-3 and C-4 of the glycogen, that minor reactions other than those in the sequence might have occurred in the enzyme preparation, that contaminants were possibly present in the glucose from the glucose 6-phosphate despite the care taken in its isolation, and that there is some limitation in the specificity of the degradative procedure. In any event, incorporation of ^{14}C was into the carbons predicted and into no other carbons except for the 7% of ^{14}C in C-1 when [2,3-^{14}C]pentose 5-phosphate was substrate.

While using subcellular preparations may allow demonstration that in the presence of certain enzymes, cofactors, and substrates, products are formed in keeping with a proposed pathway, the test of the functioning of that pathway must rest on demonstrating its functioning in the intact cell and indeed better in the intact animal or organism.

Katz *et al.* (1955) incubated liver slices with [1-^{14}C]ribose, and the glucose that formed was isolated and degraded. The glucose was assumed to be formed from glucose 6-phosphate and hence to reflect its distribution. Carbon-14 was found primarily in C-1 and C-3 of the glucose.[3] However, rather than twice as much ^{14}C in C-1 as in C-2, there was the same percentage of ^{14}C in C-1 as in C-3 (36–44% in C-1 as compared to 36–40% in C-3 in three experiments). Hiatt (1957) administered [1-^{14}C]ribose to mice, isolated glycogens from their livers, and degraded the glucoses formed on hydrolysis of the glycogens. Glucose from glycogen was presumed to be formed via glucose 6-phosphate and therefore the distributions in the glucose to reflect that in glucose 6-phosphate. More ^{14}C in C-3 than in C-1 of the glucoses was found (incorporation into C-1 ranged from 34 to 44% and into C-3 from 38 to 52% in seven experiments, with less than 7% in any of the other carbons).

The results of Katz *et al.* (1955) and Hiatt (1957) have been viewed as evidence against the functioning of the nonoxidative portion of the classical pathway (Williams *et al.*, 1983). However, the prediction that C-1 should exceed C-3 assumes that the transaldolase and transaldolase catalyzed reactions are unidirectional and that the three pentose 5-phosphates, i.e., ribose 5-phosphate, xylulose 5-phosphate, and ribulose 5-phosphate, are isotopically equilibrated. The pentose 5-phosphates need not equilibrate, and the nonoxidative portion of the pathway is completely reversible and

[3] There was as much as 6% of the ^{14}C in other carbons of the glucose, and in one glucose there was 11% in C-4. But the [1-^{14}C]glucose and [6-^{14}C]glucose used were found on degradation to have 2–5% of their ^{14}C in carbons other than those designated as bearing the label.

can serve for the synthesis of pentose 5-phosphate, i.e., 2 fructose 6-phosphate + triose 3-phosphate → 3 pentose 5-phosphate. Thus, if a relatively unlabeled pool of xylulose 5-phosphate formed from unlabeled fructose 6-phosphate and glyceraldehyde 3-phosphate reacts with [1-^{14}C]ribose 5-phosphate, [3-^{14}C]fructose 6-phosphate would result via [3-^{14}C]sedoheptulose 7-phosphate as an intermediate (see Scheme 2). In general, incorporation into C-1 relative to C-3 of hexose 6-phosphate formed in the cycle depends on the rate of ribose 5-phosphate formation in the nonoxidative portion of the pathway, the rate of ribose 5-phosphate conversion to nucleotides, and the rates of randomization in the transketolase and transaldolase catalyzed reactions (Katz and Rognstad, 1967).

Therefore, while via the nonoxidative portion of the classical pathway, ^{14}C from [1-^{14}C]ribose should be localized to C-1 and C-3 of hexose 6-phosphate, the C-1/C-3 ratio should depend on experimental conditions. Hiatt (1957) was able to obtain equal labeling in C-1 and C-3 of the glucose units of mouse liver glycogen in one experiment and more in C-1 than C-3 in another experiment by increasing the quantity of ribose administered along with [1-^{14}C]ribose. When he gave [1-^{14}C]xylose to each of two mice, 64 and 65% of ^{14}C in the glucose from glycogen was in its C-1 and 10–15% in its C-3. Assuming the xylose was converted to xylulose 5-phosphate, the greater incorporation into C-1 than into C-3 would be in accord with nonequilibration of pentose 5-phosphate, i.e., [1-^{14}C]xylulose 5-phosphate via [1-^{14}C]sedoheptulose 7-phosphate yielding [1-^{14}C]fructose 6-phosphate.

Rognstad and Katz (1974) incubated [1-^{14}C]xylitol with hepatocytes from hamsters for from 2.5 to 45 min and hepatocytes from rats for 45 min. Glucose in the cells and medium from each incubation was isolated and degraded. Irrespective of the duration of incubation, about 90% of the ^{14}C in glucose was in its C-1 and C-3, and the ratio of specific activities of C-1 to C-3 was slightly under two.

Between 1955 and 1970, a large number of studies were done in which [2-^{14}C]glucose was either administered to rats *in vivo* or perfused or incubated with various animal tissue preparations. Glucose from glycogen in the tissues was then isolated and degraded. The data are recorded in Table 12.1. If the cycle is unidirectional and fructose 6-phosphate is in complete isotopic equilibration with glucose 6-phosphate, the ratio of C-1/C-3 in the glucose from glycogen, as already noted, should be about 2. This accepts the vast amount of evidence that glucose units are formed from glucose 6-phosphate without randomization of carbons. The reason for the "about 2" is that glucose 6-phosphate from [2-^{14}C]glucose, with a ratio of 2 in one turn of the pentose cycle, if it undergoes a second turn of the cycle, yields glucose 6-phosphate with a different distribution. ^{14}C in C-1 of the glucose 6-phosphate in the second turn would be evolved as $^{14}CO_2$, ^{14}C in C-2 would be randomized to C-1 and C-3, and ^{14}C in C-3 would be randomized to C-2 of

the glucose 6-phosphate that then formed. The average number of turns of the cycle the molecules of glucose 6-phosphate will experience will depend on the rates of utilization of glucose 6-phosphate via the pentose cycle relative to the Embden–Meyerhof pathway (Katz and Wood, 1960; Wood *et al.*, 1963). Unless the pentose cycle contribution to overall metabolism is large, the ratio, however, remains about two. Thus, if metabolism were solely via the pentose cycle, the ratio would be about 1.3, while, if 10% of metabolism was via the cycle, it would be 1.8.

The C-1/C-3 ratios experimentally observed (Table 12.1) are in good agreement with those expected of the classical cycle, taking into account the reversibility of the nonoxidative portion of the pathway. Where the pathway would be expected to be particularly active, as in adipose tissue, thyroid, adrenal, and regenerating liver, since there is a need for relatively large amounts of NADPH for synthetic processes, incorportions into C-1 and C-3 are largest. Most of the distributions shown in Table 12.1 are the averages of two or more distributions obtained under the same conditions and reported in the references. In a few cases, the individual distributions differ considerably from predictions for the pathway. This is most marked for the first listing where the values of 14.3, 100, and 3.3 are means for two distributions of 19.8, 100, 1.8 and 8.9, 100, and 4.7. Whether the first represents an error in degradation, contaminant(s) in the glucose degraded, nonisotopic equilibrations in the nonoxidative portion of the cycle associated with animal variation, and/or the participation of another pathway is uncertain.

Wood *et al.* (1965) perfused mammary glands of cows with [2-^{14}C]glucose and isolated and degraded the galactose from the lactose that formed. The distributions of ^{14}C in the carbons of the galactoses relative to C-2 set to 100 were as follows:

Perfusion	C-1	C-2	C-3	C-4	C-5	C-6
1	30.6	100	18.8	0.7	4.9	0.7
2	37.0	100	24.6	0.6	1.1	1.1

Galactose from lactose is formed from glucose 6-phosphate via glucose 1-phosphate, uridine diphosphoglucose, and uridine diphosphogalactose. Therefore, the distribution of ^{14}C in the galactose should reflect the distribution of ^{14}C in the glucose 6-phosphate in the cow mammary gland. The distributions are in excellent agreement with those to be expected for the classical pathway. The relative large amounts of ^{14}C in C-1 and C-3 indicate considerable pentose cycle activity in keeping with the need for NADPH for lipid synthesis in the mammary gland.

TABLE 12.1

Distributions of ^{14}C in the Glucoses from Glycogens Formed from [2-^{14}C]Glucose *in Vivo* and *in Vitro*[a,b]

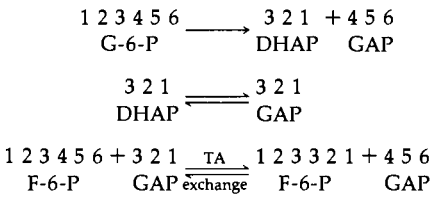
Tissue	Comments	^{14}C in C-1	C-2	C-3	C-4	C-5	C-6	Sources
				in Vivo				
Rat, liver	Fasted	14.3	100	3.3	2.4	8.4	6.3	Siu and Wood (1959)
	Regenerating	35	100	21	32	28	20	Horecker *et al.* (1958)
	Fasted	5.2	100	3.2	0.7	4.7	2.5	Hostetler and Landau (1967)
	Fasted	24.8	100	6.5	6.5	8.8	4.4	Marks and Feigelson (1957)
	Fed	9.2	100	5.4	5.4	6.2	4.3	Marks and Feigelson (1957)
Rat, muscle	Diaphragm	1.9	100	3.0	0.8	1.2	0.9	Hostetler and Landau (1967)
	Skeletal	1.6	100	2.6	0.9	4.0	1.2	Hostetler and Landau (1967)
	Skeletal, fasted	0.4	100	0.2	0.3	0.3	0.3	Marks and Feigelson (1957)
	Skeletal, fed	3.1	100	1.6	1.5	1.5	1.5	Marks and Feigelson (1957)
Rat, muscle	Heart	2.1	100	2.9	0.6	2.9	0.4	Hostetler and Landau (1967)
Rat, thyroid		6.1	100	12.4	0.7	4.0	2.4	Hostetler and Landau (1967)
Rat, kidney		7.2	100	8.0	0.8	9.7	2.6	Hostetler and Landau (1967)
Rat, brain		5.4	100	5.5	1.3	5.1	1.9	Hostetler and Landau (1967)
				in Vitro				
Rat, adipose		15.2	100	12.8	1.9	13.9	2.9	Landau and Katz (1964)
	Insulin	30.7	100	17.9	3.5	14.8	5.3	Landau and Katz (1964)

Goosefish, islets	Glucose 25/mg/dl	11.1	100	8.3	1.3	15.0	1.7	Hostetler *et al.* (1966)
	Glucose 100/mg/dl	6.4	100	4.5	1.9	20.2	1.6	Hostetler *et al.* (1966)
	Glucose 200/mg/dl	4.7	100	4.8	0.8	17.7	0.9	Hostetler *et al.* (1966)
Human intestine	Mucosal strips	10.7	100	8.3	1.7	10.1	2.8	White and Landau (1965)
Cow, adrenal		15.7	100	7.9	1.4	11.0	2.1	Weaver and Landau (1963)
	ACTH	17.0	100	9.3	2.2	9.2	2.2	Weaver and Landau (1963)
Cow, thyroid		6.6	100	5.0	0.9	9.6	1.1	Merlevede *et al.* (1963)
	TSH, 30 min	19.3	100	13.5	2.2	10.3	2.1	Merlevede *et al.* (1963)
	TSH, 120 min	8.4	100	5.9	2.0	12.1	1.1	Merlevede *et al.* (1963)
Monkey, brain	Brain stem	10.7	100	3.0	0.6	6.1	2.0	Hostetler *et al.* (1970)
	Cerebellum	12.8	100	5.7	0.7	6.0	0.9	Hostetler *et al.* (1970)
	Hemispheres	10.0	100	4.7	0.7	6.4	1.0	Hostetler *et al.* (1970)
	Hypothalmus	11.6	100	3.8	1.0	7.5[c]	1.3	Hostetler *et al.* (1970)
Mice, muscle	Heart (infant)	4.6	100	5.7		28.3[c]		Green and Landau (1965)
	Heart (adult)	3.4	100	5.2		30.9[c]		Green and Landau (1965)
	Abdominal (infant)	0.6	100	1.9		16.7[c]		Green and Landau (1965)
	Abdominal (adult)	0.1	100	1.2		19.8[c]		Green and Landau (1965)
	Diaphragm (infant)	0.7	100	2.2		19.6[c]		Green and Landau (1965)
	Diaphragm (adult)	1.0	100	1.4		10.2[c]		Green and Landau (1965)

[a] Modified from Table 1 in Landau and Wood (1983a).

[b] Activity in C-2 has been set to 100, and activities in the other carbons are relative to the activity in C-2. Where more than one experiment was done under the same conditions, the average of the activities in each carbon is recorded.

[c] Sum in C-4, C-5, and C-6.



Scheme 3.

Rognstad *et al.* (1982) incubated hepatocytes with [3-^{14}C]xylulose and [3-^{14}C]xylitol for 30–60 min. The glucose that formed was isolated and degraded. Incorporations (Table 12.2) are also in accord with metabolism by the classical pathway. [3-^{14}C]Xylitol would be expected to be metabolized via [3-^{14}C]xylulose 5-phosphate. Since C-3 of pentose 5-phosphate forms C-4 of glucose 6-phosphate in the nonoxidative portion of the classical pathway (Scheme 2), one would expect ^{14}C primarily in C-4 of glucose, as is the case. [1-^{14}C]Glyceraldehyde 3-phosphate would also be formed via the pathway, and gluconeogenesis would result in the formation of [3,4-^{14}C]glucose 6-phosphate and hence [3,4-^{14}C]glucose. Further, via the classical pathway, there would be some randomization of C-3 into C-2 and from C-2 lesser amounts into C-1 as was observed.

Incorporations of ^{14}C from [2-^{14}C]glucose into C-4, C-5, and C-6 of glucose 6-phosphate should not occur via the pentose cycle and Embden–Meyerhof pathway. When gluconeogenesis occurs, as in liver, the formation of glucose 6-phosphate via the condensation of dihydroxyacetone 3-phosphate with glyceraldehyde 3-phosphate can result in the labeling of C-4, C-5, and C-6. Transaldolase exchange can account for such incorporation (Table 12.1) whether or not gluconeogenesis occurs (Scheme 3). Indeed, the incorporation of ^{14}C from [2-^{14}C]glucose into C-4, C-5, and C-6 of glucose from glycogen has been used to estimate the extent of transaldolase exchange in adipose tissue (Landau and Bartsch, 1966).

In summary, there is an extensive literature beginning in the 1950s bearing on the tracing with ^{14}C-labeled sugars of the pentose pathway, and the findings are in accord with the classical pathway.

L-Type Pathway

Williams *et al.* (1971b) infused livers of rabbits with [2-^{14}C]glucose. Each liver was infused with the glucose over a period of 15 seconds, and then the hepatic vein of the rabbit was clamped to block the flow of blood to the liver. At 1-min intervals, termed "holding times," the livers were removed, glucose 6-phosphate from each liver isolated, and glucose from the glucose

TABLE 12.2

^{14}C Distributions in Glucose Formed from [3-^{14}C]Xylulose or [3-^{13}C]Xylitol by Rat Hepatocytes

Substrate (10 m*M*)	glucose	glucose	xylitol	xylitol	xylitol	xylitol	xylitol	xylitol	xylitol
Labeled		[3-^{14}C]Xylulose			[3-^{14}C]Xylitol				[3-^{14}C]Xylitol
PMS[a] (μM)	0	50	0	50	0	10	25	50	0
Glucose carbon no.	Relative specific activities								
C-1	4.6	15.1	1.5	6.3	1.9	4.2	5.2	5.5	0.9
C-2	18.4	37.0	7.3	21.1	9.2	18.2	20.6	22.0	6.8
C-3	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
C-4	193.0	521.0	382.0	446.0	465.0	375.0	357.0	408.0	419.0
C-5	2.5	2.6	3.8	2.7	2.6	4.1	4.6	5.8	0.9
C-6	0.8	2.1	1.9	2.2	1.3	2.0	1.8	2.1	0.6

[a] Phenazine methosulfate (PMS) was added to stimulate glucose utilization by the pentose pathway.

6-phosphate purified and degraded. Concerns have been expressed as to how closely a liver without circulation can be considered to reflect an *in-vivo* circumstance (Landau, 1981), despite measurements of the biochemical responses of the liver to hypoxia (Williams, 1981; Williams *et al.*, 1971a, 1973). The distribution of ^{14}C found in the glucoses from the glucose 6-phosphates were as follows:

Holding time (min)	C-1	C-2	C-3	C-4	C-5	C-6
1.0	5.5	44.0	3.3	6.2	6.7	34.3
2.0	20.0	27.8	13.0	6.3	0.0	32.3
3.0	2.9	63.5	3.4	12.7	8.3	9.2
4.0	5.8	67.0	0.0	3.1	5.6	16.5
5.0	5.7	36.5	0.0	5.6	11.4	40.8

There is marked variation in the incorporations into the carbons from minute to minute. Particularly noteworthy are extensive incorporations into C-6, at 1, 2, and 5 min with lesser incorporations at 3 and 4 min. Williams *et al.* (1971b) infused [2-^{14}C]glucose into one rabbit liver at 5-min intervals for 15 min and then isolated and degraded the glucose from glucose 6-phosphate. Again there was incorporation particularly into C-6, i.e., C-1 = 6.9, C-2 = 13.3, C-3 = 2.7, C-4 = 17.4, C-5 = 2.5, and C-6 = 57.2. Williams and Clark (1971) also reported that Schofield *et al.* (1970) perfused a rabbit liver with [2-^{14}C]glucose, and after 2 hr, the distribution in glucose from glucose 6-phosphate was C-1 = 4.6, C-2 = 8.2, C-3 = 1.2, C-4 = 10.0, C-5 = 6.9, and C-6 = 69.1. Thus, again there was a marked movement of C-2 of glucose into C-6 of glucose 6-phosphate.

When Williams *et al.* (1971b) infused [1-^{14}C]ribose into a liver from a fasted rabbit for 15 seconds and then, after a holding period of 5 min, degraded the glucose from glucose 6-phosphate, ^{14}C was localized in particular into C-1 and C-3 of the glucose, in keeping with the findings of Hiatt (1957) and of Katz *et al.* (1955), although there was a greater concentration of ^{14}C in C-1, i.e., C-1 = 64.8, C-2 = 7.2, C-3 = 10.0, C-4 = 4.4, C-5 = 6.0, C-6 = 7.6. Williams *et al.* (1978b) also incubated a rat liver enzyme preparation with [1-^{14}C]ribose as Horecker *et al.* (1954) had done. The distributions of ^{14}C in the glucose carbons from the glucose 6-phosphates formed were as follows:[4]

[4] Small differences from these distributions are found in distributions reported by Williams and Clark (1971).

Incubation time	1 min	2 min	5 min	30 min	3 hr	8 hr	17 hr
C-1	1.0	0.5	1.1	2.0	28.2	40.6	57.2
C-2	45.0	44.0	16.1	12.9	20.9	13.4	10.4
C-3	1.7	0.1	2.2	1.9	9.8	16.3	25.1
C-4	10.1	3.1	4.9	6.9	4.5	4.6	2.7
C-5	0.9	5.9	0.9	0.1	1.9	3.7	1.4
C-6	41.3	46.4	74.8	76.2	34.7	21.4	3.4

After 17 hr of incubation, the distribution is similar to that found by Horecker *et al.* (1954). However, at earlier times, mostly in the first 30 min, there are large percentages of ^{14}C in C-6 as well as in C-2 of the glucose 6-phosphates.

Williams (1980) concluded that the reactions of the classical pentose pathway cannot be reconciled with these incorporations and therefore that a new and different sequence of reactions must exist to produce such labeling in liver.

Gerdes *et al.* (1974) reported results of perfusions of normal and regenerating rabbit liver with [2-^{14}C]glucose for 2 hr and then isolation of glucose 6-phosphate and ribose 5-phosphate and their degradation. The percentage distributions are listed in Table 12.3. They concluded from these data that the larger amounts of ^{14}C appearing in C-6 of glucose 6-phosphate and in C-5 of ribose 5-phosphate, while prohibited by the operation of the classical pentose phosphate pathway, are diagnostic of the operation of an alternate pathway. The quantities of ^{14}C in C-6 of glucose 6-phosphate from regenerating livers, 8.5–17.9%, are generally less than those reported in their perfusions of normal liver. The distributions in the glucose 6-phosphate from liver regenerating 96 hr after partial hepatectomy has ^{14}C localized primarily to C-2 with about twice as much ^{14}C in C-1 as C-3, 13.2 and 6.2%, in accord with the classical pathway.[5]

While the classical pathway will not account for the incorporation into C-6 of glucose 6-phosphate, neither will the L-type pathway (Chapter 2, Fig. 2.3) proposed as the alternative.[6] Williams (1980) has concluded that the incorporation into C-6 occurs not by the L-type pathway but by a series of exchange reactions catalyzed by transaldolase and transketolase. Wil-

[5] While many of the initial observations of Williams and his co-workers were made using rabbit liver, the studies with [2-^{14}C]ribose using a rat liver preparation gave similar results, and as we shall see, their later experiments were with rat hepatocytes. Also Williams *et al.* (1978b) found similar enzyme patterns in rat and rabbit liver preparations.

[6] The "L" stands for liver, and Williams (1980) refers to the classical pathway as the "F" pathway, since he finds that the classical pathway only functions in adipose tissue.

TABLE 12.3

Distribution of ^{14}C in Glucose 6-Phosphate and Ribose 5-Phosphate Isolated from Normal and Regenerating Liver Perfused with [2-^{14}C]Glucose

	Nonregenerating liver control (%)	Time after 70% partial hepatectomy (hr)			
		12	24	48	96
Glucose 6-phosphate					
C-1	12.1	16.6	15.1	10.5	13.2
C-2	10.4	51.8	57.8	60.7	63.5
C-3	2.0	5.3	4.8	4.2	6.2
C-4	21.3	2.0	2.5	3.7	1.6
C-5	18.7	6.3	9.5	11.7	7.1
C-6	35.5	17.9	10.0	9.1	8.5
Ribose 5-phosphate					
C-1	15.1	21.6	37.8	28.3	33.5
C-2	33.5	8.6	24.8	16.8	20.8
C-3	8.6	6.8	5.1	6.9	9.0
C-4	15.5	20.7	13.4	9.3	18.5
C-5	24.3	42.1	18.9	38.6	18.2

liams and Clark (1971) postulated that [1-^{14}C]xylulose 5-phosphate formed from [2-^{14}C]glucose reacts as follows: [1-^{14}C]xylulose 5-phosphate + erythrose 4-phosphate → [1-^{14}C]fructose 6-phosphate + glyceraldehyde 3-phosphate; [1-^{14}C]sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate → [1-^{14}C]fructose 6-phosphate + erythrose 4-phosphate; [3-^{14}C]-glyceraldehyde 3-phosphate can be obtained from [1-^{14}C]fructose 6-phosphate by glycolysis; then by transaldolase exchange [3-^{14}C]glyceraldehyde 3-phosphate + fructose 6-phosphate → [6-^{14}C]fructose 6-phosphate + glyceraldehyde 3-phosphate. [1,3-^{14}C]Glyceraldehyde 3-phosphate is also formed via the condensation of [1-^{14}C]ribose 5-phosphate with [1-^{14}C]xylulose 5-phosphate. Therefore, Williams and Clark (1971) also postulated that [1,3-^{14}C]glyceraldehyde 3-phosphate + fructose 6-phosphate → [4,6-^{14}C]fructose 6-phosphate + glyceraldehyde 3-phosphate. Thus, [1-^{14}C]fructose 6-phosphate, [4,6-^{14}C]fructose 6-phosphate, and [6-^{14}C]fructose 6-phosphate are formed in equilibrium reactions, but ^{14}C is found localized particularly to C-6 in glucose 6-phosphate. A modified scheme for the conversion of [2-^{14}C]fructose 6-phosphate to [2,6-^{14}C]fructose 6-phosphate has also been proposed (Williams *et al.* 1978b). Since these reactions are neither those of the L-type pathway nor the classical pathway, they provide no support for the existence of either pathway.

In all cases where others have incubated [2-^{14}C]glucose, incorporation has not been found into C-6 of glucose from glycogen (Table 12.1) and, as

we will see, into glucose 6-phosphate. Further, when Longenecker and Williams (1980b), incubated hepatocytes with [2-^{14}C]glucose, the glucose 6-phosphate isolated had negligible ^{14}C in its C-6, i.e., C-1 = 2.5, C-2 = 66.8, C-3 = 4.2, C-4 = 1.8, C-5 = 21.8, and C-6 = 2.9.

Longenecker and Williams (1980b) reported crucial evidence for the L-type pathway in their incubations of hepatocytes with [5-^{14}C]glucose and [4,5,6-^{14}C]glucose. Glucose 6-phosphate was isolated from the cells after 1 hr of incubation and the glucose from the glucose 6-phosphate degraded. The results are shown in Table 12.4 and should be considered in terms of distributions to be expected via the L-type pathway (Chapter 2, Fig. 2.3). In the pathway, three molecules of glucose 6-phosphate are oxidized to three molecules of CO_2 and three of pentose 5-phosphate, i.e., the oxidative portion is the same as in the classical pathway. However, the three molecules of pentose 5-phosphate are converted to one molecule of glucose 6-phosphate, one of fructose 6-phosphate, and one of dihydroxyacetone 3-phosphate. The distributions in these products of the carbons of glucose, again designated 1–6, are as follows (Williams *et al.* 1978a; Williams, 1980):

$^{4\,2\,3\,4\,5\,6}$ glucose 6-phosphate $^{6\,5\,3\,4\,5\,6}$ fructose 6-phosphate $^{2\,3\,2}$ dihydroxyacetone 3-phosphate

Longenecker and Williams (1980b) found ^{14}C from [5-^{14}C]glucose in C-2 of glucose 6-phosphate (Table 12.4), which is in accord with the pathway assuming [2-^{14}C]fructose 6-phosphate is formed and is isomerized to [2-^{14}C]glucose 6-phosphate. There was 17.7% of the ^{14}C in the glucose 6-phosphate in its C-2 and 70.8% in its C-5. The source of the 11.5% in C-4 is unexplained. Incorporation of ^{14}C into C-2 cannot be attributed to the L-type pathway without first eliminating incorporation via (1) gluconeogenesis from [2-^{14}C]glyceraldehyde 3-phosphate formed via the classical

TABLE 12.4

Distribution of ^{14}C in [5-^{14}C]Glucose and [4,5,6-^{14}C]Glucose Incubated with Liver Cells and in Glucose 6-Phosphate Then Isolated from the Cells

A. [5-^{14}C]Glucose					
C-1	C-2	C-3	C-4	C-5	C-6
0.0	1.3	0.9	2.1	94.0	1.7 (substrate)
0.6	17.7	1.9	11.5	70.8	1.4 (glucose 6-phosphate)
B. [4,5,6-^{14}C]Glucose					
C-1	C-2	C-3	C-4	C-5	C-6
1.2	1.6	1.7	33.0	31.4	31.0 (substrate)
2.0	12.1	2.7	30.0	24.3	28.7 (glucose 6-phosphate)

pathway and/or (2) futile cycling, i.e., [5-^{14}C]glucose 6-phosphate → [5-^{14}C]fructose 6-phosphate → [5-^{14}C]fructose 1,6-diphosphate → dihydroxyacetone 3-phosphate + [2-^{14}C]glyceraldehyde 3-phosphate, then isotopic equilibration catalyzed by triose-P isomerase to form [2-^{14}C]dihydroxyacetone 3-phosphate and [2-^{14}C]dihydroxyacetone 3-phosphate + [2-^{14}C]glyceraldehyde 3-phosphate → [2,5-^{14}C]fructose 1,6-diphosphate, and hence [2,5-^{14}C]glucose 6-phosphate.

Longenecker and Williams (1980b) eliminated incorporation of the ^{14}C from [5-^{14}C]glucose into C-2 of glucose 6-phosphate by these routes because of their result with [4,5,6-^{14}C]glucose. If [4,5,6-^{14}C]glucose is metabolized via futile cycling and/or gluconeogenesis, the glucose 6-phosphate formed should have the same amounts of ^{14}C in C-1, C-2, and C-3 (except for small changes in their relative quantities, assuming a small classical pentose pathway in liver). Instead they find ^{14}C in C-2 and not in C-1 and C-3 (Table 12.4). However, C-4 of glucose would rearrange to C-1 of glucose 6-phosphate and C-6 rearrange to C-1 of fructose 6-phosphate via the L-type pathway, and C-4 and C-6, as well as C-5, of the substrate glucose were labeled with ^{14}C. Thus, if [4,5,6-^{14}C]glucose were metabolized by the L-type pathway, there should have been twice as much ^{14}C in C-1 as in C-2 of glucose 6-phosphate, assuming complete equilibration of fructose 6-phosphate with glucose 6-phosphate (Katz, 1981).

Williams has attempted to explain the lack of incorporation in C-1 in two ways. First, Williams (1981) proposed an explanation that included glucose 6-phosphate not equilibrating with fructose 6-phosphate. If there were not equilibration, there would be more than twice as much ^{14}C in C-1 as in C-2 of glucose 6-phosphate. When this was noted, Williams *et al.* (1983) offered a second explanation. They proposed that futile cycling at the pyruvate level results in the distributions in glucose 6-phosphate observed. They reported that [4,5,6-^{14}C]glucose forms lactate with ^{14}C "shifted from C-2 and heavily deposited with C-3 with a lighter transfer to C-1 . . . The net effect of the above on the ^{14}C labeling of C-1 and C-2 of hexose 6-phosphate using [4,5,6-^{14}C]glucose as substrate is a minimum accumulation of ^{14}C in C-2 from C-5 and an assault on C-1 by the above events." By glycolysis, [4,5,6-^{14}C]glucose should form uniformly labeled pyruvate, which by exchange in the dicarboxylic acid shuttle, i.e., pyruvate + CO_2 ⇌ oxalacetate ⇌ malate ⇌ fumarate ⇌ succinate (Topper and Hastings, 1949), could result in decreased ^{14}C in C-1 relative to C-2 and C-3 of the pyruvate and hence of lactate. But it could not cause preferential dilution of C-3 relative to C-2 of the lactate, and C-3 and C-2 of lactate become, respectively, C-1 and C-2 of glucose 6-phosphate. Williams *et al.* (1983) have also indicated that the experiments with [4,5,6-^{14}C]glucose were intended to test only whether gluconeogenesis occurred and not the functioning of the L-type pathway. However, whether intended to or not, the distri-

bution obtained with [4,5,6-^{14}C]glucose is a test of whether the pathway functions. Landau and Wood (1983a) concluded that there is no mechanism known or proposed, including the L-type pathway, by which the metabolism of [5-^{14}C]glucose and [4,5,6-^{14}C]glucose can result in the same labeling in C-1, C-2, and C-3 of glucose 6-phosphate, i.e., ^{14}C in C-2 and none in C-1 and C-3.

Williams (1981) has criticized the data on the classical pathway on the basis that he has analyzed glucose 6-phosphate and that neither glucose nor glycogen reflect the distribution in glucose 6-phosphate. He states, "I find little value in pentose cycle studies when substances (tissue glucose, glucose units of glycogen, lactate, glycerol, lactose, etc.) other than specific intermediates of the pathway are isolated, purified, and degraded." Clearly this reflects Williams's experience, since his degradations of glucose 6-phosphate give values different from those found in glucose and glucose units of glycogen. However, to believe that glucose and glycogen are not direct products of glucose 6-phosphate, i.e., without randomization of carbons in their formation, is contrary to what is known of their formation.

Rognstad *et al.* (1982) degraded both glucose and glucose from glucose 6-phosphate formed by hepatocytes incubated for 90 min with [3-^{14}C]xylitol and unlabeled dihydroxyacetone. The distributions were as follows:

	C-1	C-2	C-3	C-4	C-5	C-6
Glucose	4.9	14.4	100	328	1.6	4.3
Glucose 6-phosphate	8.2	29.5	100	472	14.6	10.8

The glucose 6-phosphate and the glucose have similar distributions. There are differences, but glucose 6-phosphate isolated from hepatocytes yields the distribution in glucose 6-phosphate at the time of incubation is terminated, while the distribution in glucose is for glucose that accumulates throughout the incubation.

Scofield *et al.* (1984) perfused rat livers for 90 min with [2-^{14}C]glucose, [3,4-^{14}C]glucose, [5-^{14}C]glucose, and [4,5,6-^{14}C]glucose. They degraded glucose from the glycogen and glucose from glucose 6-phosphate formed in the livers. In each perfusion, glucoses from the glycogen and glucose 6-phosphate had essentially the same distribution of ^{14}C. The distributions were those to be expected from the classical pentose pathway and not the L-type pathway. There was no significant incorporation of ^{14}C from [2-^{14}C]glucose into C-6 of glucose 6-phosphate or preferential incorporation of ^{14}C from [4,5,6-^{14}C]glucose into C-2 of glucose 6-phosphate.

They also perfused rat livers with [1-^{14}C]ribose for 30 and 90 min. The distributions of ^{14}C in the glucoses from glycogens in the livers and glucoses

released into the perfusate are the same as those in the glucoses from the glucose 6-phosphate from the livers. The distributions are similar to those reported by Katz *et al.* (1955) and by Hiatt (1957) and specifically without significant incorporation of ^{14}C into C-2 and C-6. Furthermore, Kuehn and Scholz (1982), using radiolabeled glucoses to measure the rates of flux through the pentose cycle in perfused liver, have found rates inconsistent with the operation of the L-type pathway.

Williams *et al.* (1983) have also concluded that "glycogen data are of little use, because it has been shown using fed and starved hepatocytes that ^{14}C-labeled glucose directly exchanges its carbon atoms into glycogen, and only when the glucose is above 20 m*M* does incorporation into glycogen commence." However, it does not matter whether there is a net synthesis of glycogen but only that the glucose is converted to glycogen via glucose 6-phosphate, as by exchange (Landau and Wood, 1983b).

Williams *et al.* (1983) appear to believe that because only a small portion of glucose appears to be metabolized by the classical pathway in liver in most conditions, this is evidence against the sequence of reactions composing that pathway. The sequence of reactions is not determined by how much is metabolized by the sequence. Under certain circumstances, randomization of ^{14}C is so little that no metabolism by the pentose pathway can be detected or the degree of randomization cannot be ascribed with any certainty to a particular pathway.

While Williams (1980) has focused his attention on liver, he has stated that of the 17 different animal and plant tissues he has examined, the classical pathway is only present in fat tissue. In incubations of adipose tissue he and his co-workers (Blackmore *et al.*, 1982) have obtained results in accord with the data obtained by others, including those recorded in Table 12.1. His procedure for identifying the L-type pathway in a tissue, in addition to showing that ribose 5-phosphate is converted to hexose 6-phosphate by an "appropriate" tissue enzyme preparation, is to show that the tissue preparation converts D-*glycero*-D-*iodo*-octulose 1,8-diphosphate and sedoheptulose 7-phosphate to hexose 6-phosphate, that there is conversion of [^{14}C]arabinose 5-phosphate to [^{14}C]hexose 6-phosphate, and that aldolase activity in the tissue is equal or greater than transaldolase and transketolase activities. However, the ability of an enzyme preparation from a tissue to make these transformations does not mean the reactions function to any significant degree in the intact cell.

Thus, while D-octulose diphosphate is found in red blood cells (Vanderheiden, 1965) and therefore presumably enzymes are there to form it, $^{14}CO_2$ is formed by mature red blood cells from [1-^{14}C]glucose but not from [6-^{14}C]glucose (Brin and Yonemoto, 1958). By the L-type pathway, but not the classical pathway, C-6 of glucose becomes C-1 of glucose 6-phosphate,

and hence $^{14}CO_2$ would be formed from [6-^{14}C]glucose if that pathway were active.

In summary, under certain conditions Williams and his co-workers obtained distributions of ^{14}C in glucose 6-phosphate on presentation of ^{14}C specifically labeled sugars to various liver preparations that are in keeping with metabolism via the classical pentose pathway. In many other circumstances they observed distributions that are not consistent with any known or proposed pathway, including the proposed L-type pathway. There is no report other than that from Williams and his co-workers of such distributions. The results of Williams and his co-workers could be a consequence of impure glucose 6-phosphate, of limitations in the degradative procedure, or of an impurity in the labeled substrates despite the care taken. This is particularly so in the studies at early time periods when small percentages of the doses of labeled sugars are recovered in glucose 6-phosphate (Williams *et al.*, 1978b).[7] It is of course possible that there are reactions that account for these distributions, but if so, the relationship of such reactions to the pentose pathway is not evident.

Other Formulations

Three other formulations of the nonoxidative branch of the pathway that have been proposed are discussed in Chapter 2, with the schemes depicted in Figs. 2.4, 2.5, and 2.6 of that chapter. The rearrangement of carbons predicted from the schemes are shown in Figs. 12.1, 12.2, and 12.3.

In the scheme proposed by Sable (1966), (Fig. 12.1), two molecules of xylulose 5-phosphate and one of ribose 5-phosphate formed in the oxidative branch, yield one molecule of fructose 6-phosphate bearing carbons 233456, one of fructose 1,6-diphosphate bearing carbons 232456, and one of glyceraldehyde 3-phosphate bearing carbons 456. The only overall difference from the classical pathway then is that fructose 1,6-diphosphate rather than fructose 6-phosphate containing carbons 232456 is formed. Therefore, tracing with labeled carbon cannot differentiate between the two schemes, unless one assumes that fructose 1,6-diphosphate does not isotopically equilibrate with fructose 6-phosphate. Where there is no, or minimal, fructose 1,6-diphosphatase activity, there would be no, or minimal, incorporation of ^{14}C from [2-^{14}C]glucose into C-3 of fructose 6-phosphate and hence glucose 6-phosphate in Sable's scheme. However, since the transketolase and aldolase catalyzed reactions of the scheme are reversible, incor-

[7] Horecker *et al.* (1954) did not find sufficient glucose 6-phosphate for degradation when they incubated [1-^{14}C]ribose 5-phosphate for brief periods with the rat liver enzyme preparation (see discussion in Horecker *et al.*, 1982).

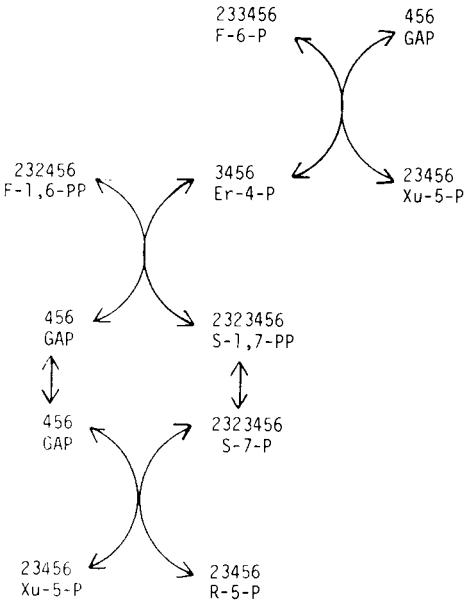


Fig. 12.1. Rearrangement of carbons in alternative nonoxidative branch. From Sable (1966) *Adv. Enzymol.* Copyright © 1966. John Wiley & Sons, Ltd. Reprinted by permission of John Wiley & Sons, Ltd.

poration can occur into C-3 of hexose 6-phosphate, i.e., [2-^{14}C]fructose 6-phosphate + glyceraldehyde 3-phosphate → [2-^{14}C]xylulose 5-phosphate + erythrose 4-phosphate. Then [2-^{14}C]pentose 5-phosphate in the forward direction would yield label in C-3 of fructose 6-phosphate. Thus, tracers at present cannot rule out the scheme of Sable (1966).

The scheme proposed for muscle by Wood (1974c) is overall in the forward direction (Fig. 12.2) ribose 5-phosphate + 2 xylulose 5-phosphate → fructose 6-phosphate + 2 glyceraldehyde 3-phosphate + dihydroxyacetone 3-phosphate, with the distributions of carbons in the products, respectively, 233456, 456, and 232. If one molecule of glyceraldehyde 3-phosphate and one of dihydroxyacetone 3-phosphate reform hexose 6-phosphate, the distribution would be 232456. Again, with present tracings, this scheme cannot be differentiated from the classical pathway in tissues where there is gluconeogenesis. Where there is not gluconeogenesis, as in muscle, the scheme in the forward direction could not yield ^{14}C from [2-^{14}C]glucose in C-3 of hexose 6-phosphate. However, reversal could again yield such incorporation, i.e., [2-^{14}C]fructose 6-phosphate +

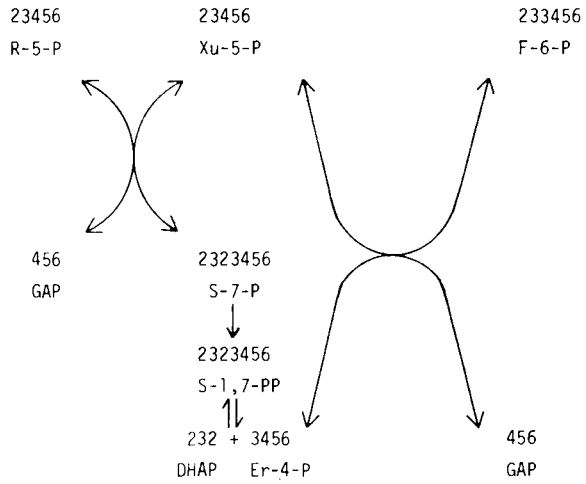


Fig. 12.2. Rearrangement of carbons in alternative nonoxidative branch in muscle. From Wood (1974c). Reprinted by permission from *Biochem. J.*, **138**, 71–76. Copyright © 1974. The Biochemical Society, London.

glyceraldehyde 3-phosphate → [2-^{14}C]xylulose 5-phosphate + erythrose 4-phosphate.

The scheme proposed by Severin and Stepanova (1981) (Fig. 12.3) has in the forward direction the same overall balance as that of Wood (1974c): ribose 5-phosphate + 2 xylulose 5-phosphate → fructose 6-phosphate + 2 glyceraldehyde 3-phosphate + dihydroxyacetone 3-phosphate. Glucose 6-phosphate is both a reactant and product of the scheme, but the carbon distributions of the glucose 6-phosphates are the same, i.e., they are both 123456. Therefore, glucose 6-phosphate does not appear in the net equation. The distributions of carbons in the fructose 6-phosphate, glyceraldehyde 3-phosphate, and dihydroxyacetone 3-phosphate are the same as in the scheme of Wood (1974c). Hence, the same considerations apply. That is, in the forward direction, gluconeogenesis would also have to occur to account for incorporation of ^{14}C of [2-^{14}C]glucose into C-3 of glucose 6-phosphate.

Reversal of the reactions would not be expected to yield fixed ratios. In tissues, such as adipose tissue, thyroid, adrenal, and brain, incorporations of ^{14}C for [2-^{14}C]glucose into C-1 and C-3 of glucose 6-phosphate derivatives are relatively high, and the ratios of incorporations approach those predicted for the classical scheme. Probably, where net flow is large in the

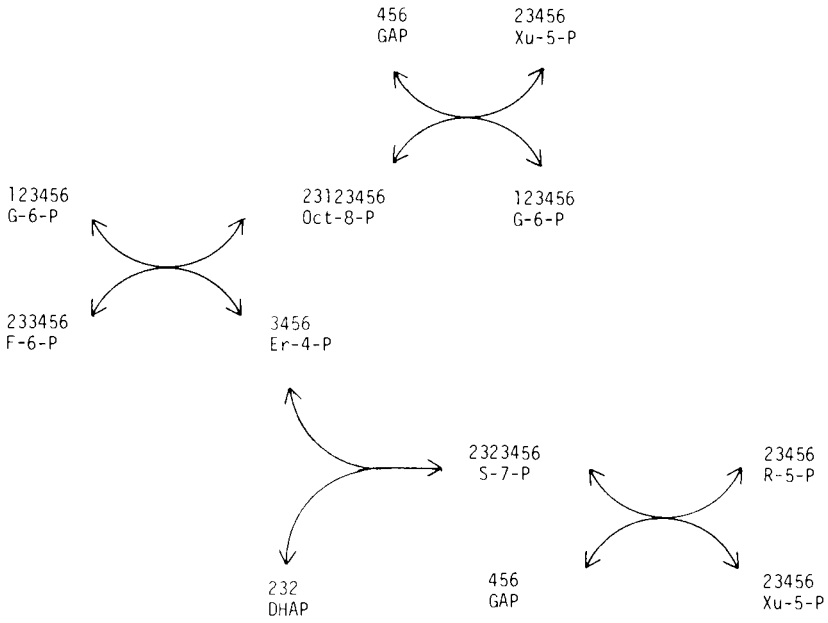


Fig. 12.3. Rearrangement in alternative nonoxidative branch. From Severin and Stepanova (1981). Reprinted with permission from *Adv. Enzyme Regul.* **19**, Severin and Stepanova, Copyright (1981), Pergamon Press Ltd.

forward direction, the contribution by reversal is masked. Thus, the ratios, near 2, seem to be against these alternative schemes. However, where C-1/C-3 ratios are above 2, these schemes, rather than lack of isotopic equilibration of the pentose phosphates, at least based on studies with isotopes, remain particular possibilities.

QUANTITATION

Various approaches can and have been used to estimate the contribution of the classical pentose pathway to glucose utilization by various tissues and in the whole animal, and under various physiological and pathological conditions. Estimates using these approaches are presented in Chapter 8.

The distribution of ^{14}C from [2-^{14}C]glucose in glucose 6-phosphate or a substance reflecting that distribution, such as glucose from glycogen, has in particular been used (see Table 12.1). Glycogen is relatively easy to isolate and purify. For the estimations, it is usually assumed that glucose is metabolized via the Embden–Meyerhof pathway, the pentose cycle, and nontriose

phosphate pathways (such as metabolism to glycogen, nucleic acids, and glucuronic acid[8]) and that there is complete isotopic equilibration of fructose 6-phosphate with glucose 6-phosphate (Wood *et al.* 1963). The glucose 6-phosphate will bear ^{14}C in its C-2 to the extent glucose is utilized by all the pathways. If there is no pentose cycle contribution, the ^{14}C will remain solely in C-2. To the extent the cycle contributes relative to the total quantity of glucose utilized, ^{14}C, as noted, will be randomized into C-1 and C-3 of glucose 6-phosphate. It can be shown, designating the specific activities of the first three carbons of glucose 6-phosphate by C-1, C-2, and C-3 and defining PC as the fraction of glucose utilized that is metabolized in the cycle, i.e., the quantity of glucose metabolized to CO_2 and glyceraldehyde 3-phosphate, that $C\text{-}1/C\text{-}2 = 2PC/(1 + 2\ PC)$ and $C\text{-}3/C\text{-}2 = PC/(1 + 2\ PC)$ (Katz and Wood, 1960). Similar relationships can be derived if [3-^{14}C]glucose rather than [2-^{14}C]glucose is used.

The assumption of complete equilibration of fructose 6-phosphate with glucose 6-phosphate is supported by the relatively high activity of phosphohexose isomerase in tissues. To the extent there is incomplete equilibration, the C-1/C-2 and C-3/C-2 ratios in fructose 6-phosphate will exceed those in glucose 6-phosphate. Thus, if the distributions of label are determined in glucose 6-phosphate or its equivalent, such as glycogen, and in fructose 6-phosphate or its equivalent, dihydroxyacetone 3-phosphate, the extent of equilibration can be estimated. Using this method, equilibration in adipose tissue has been found to be extensive but not complete. Expressions have been derived allowing for the estimation of PC as a function of the extent of equilibration (Landau *et al.*, 1964; Landau and Bartsch, 1966).

In deriving the expressions that have been presented, the pentose cycle is assumed to be unidirectional. As already noted, reversal of the nonoxidative portion of the cycle and nonisotopic equilibration of the pentose 5-phosphates will affect the C-1/C-2 and C-3/C-2 ratios in glucose 6-phosphate (Katz and Rognstad, 1967). From the C-1/C-2 and C-3/C-2 ratios, maximum and minimum values for PC can then be estimated.

Hakim *et al.* (1976) introduced another method for estimating the pathway's contribution. They administered ^{14}C-labeled sodium gluconate to rats and thus labeled the 6-phosphogluconate pool. The amount of label in the pool and its size with time allows calculation of the 6-phosphogluconate turnover rate. When combined with an estimate of the glucose consumption

[8] Glucuronic acid formed from glucose 6-phosphate can be metabolized to pentose 5-phosphate with the formation of CO_2 from C-6 of the glucose 6-phosphate. The pentose 5-phosphate can then enter the pentose cycle. If this occurs, the glucuronic acid pathway becomes a triose phosphate–forming pathway. In adipose tissue, this pathway is so small as to have a negligible effect on the quantitation of the pentose cycle (Landau *et al.*, 1966). The glucuronic acid pathway has not been quantitated in other tissues.

rate, this yields a measure of the quantity of glucose consumed that is metabolized via the pathway.

Estimates of PC can also be made from the incorporation of ^{14}C from [1-^{14}C]glucose and [6-^{14}C]glucose (or [U-^{14}C]glucose) into a triose phosphate derivative (lactate, glycerol, and fatty acids). The expression relating the yields of ^{14}C in the derivative to PC is:

$$\frac{[1\text{-}^{14}C]\text{Glucose in triose phosphate}}{[6\text{-}^{14}C]\text{Glucose in triose phosphate}} = \frac{1 - PC}{(1 + 2\ PC)}$$

The further the product selected to estimate PC is from glucose 6-phosphate in the metabolic scheme, the more assumptions must be made (Wood *et al.*, 1963). Thus, if there is no pentose cycle contribution, equal amounts of ^{14}C from [1-^{14}C]glucose and [6-^{14}C]glucose will be incorporated into a triose phosphate derivative, such as lactate. However, if there is also no isotopic equilibration of dihydroxyacetone 3-phosphate with glyceraldehyde 3-phosphate, C-1 of glucose can only be incorporated into dihydroxyacetone 3-phosphate derivatives, such as glycerol, and C-6 into glyceraldehyde 3-phosphate derivatives, such as lactate. Thus, the assumption in using the last expression is that there is complete isotopic equilibration of dihydroxyacetone 3-phosphate with glyceraldehyde 3-phosphate. This is generally supported by the relatively high activity of triose-phosphate isomerase in tissues. By using yields of triglyceride rather than glycerol or fatty acids, one can make an estimate of PC even if there is not equilibration. One can estimate the extent of triose phosphate isomerization by measuring the C-1/C-6 ratio in both dihydroxyacetone 3-phosphate and glyceraldehyde 3-phosphate derivatives and with that estimate make a better estimate of PC. This has also been done for adipose tissue (Katz *et al.* 1966). In using triose phosphate yields, one must also assume metabolism by non-triose-phosphate pathway(s) is negligible or at least small relative to the triose phosphate–forming pathways.

Yields of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose can also be used to estimate PC. The relationship between the yields and PC is (Katz and Wood, 1963):

$$\frac{G1CO_2 - G6CO_2}{1 - G6CO_2} = \frac{3PC}{(1 + 2\ PC)}$$

However, the yields of CO_2 ($G1CO_2$ and $G6CO_2$) are specific yields, i.e., the yield of CO_2 divided by the quantity of glucose utilized. While a molecule of $^{14}CO_2$ is formed for every molecule of [1-^{14}C]glucose entering the cycle and none from [6-^{14}C]glucose, the amount of $^{14}CO_2$ formed from both via the Krebs cycle depends on the fraction of the label entering the Krebs cycle that is oxidized to CO_2. The specific yields provide a measure of this fraction.

The use of the ratio of $^{14}CO_2$ yields, rather than specific yields, from [1-^{14}C]glucose and [6-^{14}C]glucose cannot be used to quantitate pentose cycle activity. To illustrate this, although utilization of glucose is primarily via the Embden–Meyerhof pathway, the ratio is infinite in mature red blood cells, since in them there are no mitochondria. The ratio may also be less than one even though the pentose cycle is active, if sufficient C-1 of glucose is preferentially deposited in a dihydroxyacetone 3-phosphate derivative because of nonisotopic equilibration of dihydroxyacetone 3-phosphate with glyceraldehyde 3-phosphate. This has been shown to occur under certain conditions also in adipose tissue (Landau *et al.*, 1966).

In tissues where gluconeogenesis occurs, as in liver and kidney, the expressions yield estimates of PC that are further approximations. To the extent there is futile cycling, glucose $\rightleftharpoons$ glucose 6-phosphate, the expression using distributions in glucose 6-phosphate still holds so long as steady state exists and there is a large pool of substrate glucose. The glucose 6-phosphate $\rightarrow$ glucose is then the equivalent of a non-triose-phosphate pathway. However, gluconeogenesis from labeled triose phosphate results in the incorporation into C-1 and C-3 of glucose 6-phosphate from other than the molecules of glucose 6-phosphate formed in the pentose cycle.

Two simplified models have been developed to estimate the interaction of the pentose cycle with the gluconeogenic pathway in liver and preliminary data obtained in support of the models (Rognstad, 1976). Stoichiometry of the cycle is 6 glucose 6-phosphate $\rightarrow$ 5 glucose 6-phosphate + 6 CO_2. The rate of glucose-6-phosphate dehydrogenase activity is calculated relative to that of glucose 6-phosphatase. In the first model, estimates are made using yields of ^{14}C in $^{14}CO_2$ and glucose from [1-^{14}C]galactose. In the second model estimates are made from the distribution in glucose of ^{14}C from ^{14}C-labeled gluconeogenic substrates such as [1-^{14}C]propionate.

In summary, there are several approaches to estimating the contribution of the pentose pathway. The choice of method depends in part on the tissue or organism in which the pathway is to be estimated. Thus, availability of glycogen, ease of CO_2 collection, measurement of glucose utilization, etc., may be determinants. A number of assumptions are made in making an estimate, but these can usually be tested or supported through other biochemical information available with regard to the tissue or organism.

CONCLUSION

A knowledge of the amount, K_m, and V_{max} of each enzyme of a proposed pathway allows assessment of whether that pathway is likely to function. Such knowledge has been employed to help establish the existence of the

pathways of glycolysis, gluconeogenesis, glycogen formation and breakdown, the pentose cycle, etc., but the relative roles of these pathways in the intact animal cannot be determined from such data. When data on the concentrations of the intermediates in the pathways are combined with those on the concentrations of enzymes and their characteristics, estimates can be made using modeling techniques and computer technology. But this represents a formidable undertaking of questionable validity, since neither the enzymes nor the intermediates are uniformly distributed intracellularly.

There are also formidable obstacles to the making of estimates using tracers. These have been encountered when quantitating the classical pentose pathway contribution to metabolism and relative to the Embden–Meyerhof pathway using distributions of ^{14}C from [^{14}C]glucose in glucose 6-phosphate and its derivatives. Controversy over the role of L-type pathway has introduced an additional concern. Nevertheless, some solutions have been obtained.

With the exception of distributions reported by Williams and his coworkers, all data are in accord with the overall reactions of the classical pentose pathway. To date no other investigators have obtained data interpreted to be in accord with the functioning of the L-type pathway. There is agreement that in those tissues where lipogenesis is prominent, such as adipose tissue, the classical pathway functions.

The large amount of NADPH that must be generated via the cycle, resulting in the predominence of the oxidative segment of the cycle, is an ideal circumstance for quantitating the cycle using the equations based on the distributions of ^{14}C from [2-^{14}C]glucose. In most tissues, there is not a large requirement for NADPH, and the reversal of the nonoxidative segment of the cycle becomes more prominent. By this reversal, ^{14}C from ^{14}C-labeled glucoses can be redistributed, and then the standard equations when using [2-^{14}C]glucose no longer hold. Nevertheless, the randomizations of ^{14}C from [2-^{14}C]glucose into C-1 and C-3 of the glucose 6-phosphate derivatives indicating the functioning of the classical pentose pathway, or its equivalent, provide a measure of the pathway's activity, and such randomizations have been observed in many tissues.

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