Energy Transformation in Biological Systems

Ciba Foundation Symposium 31 (new series)

In tribute to Fritz Lipmann on his 75th birthday

1975

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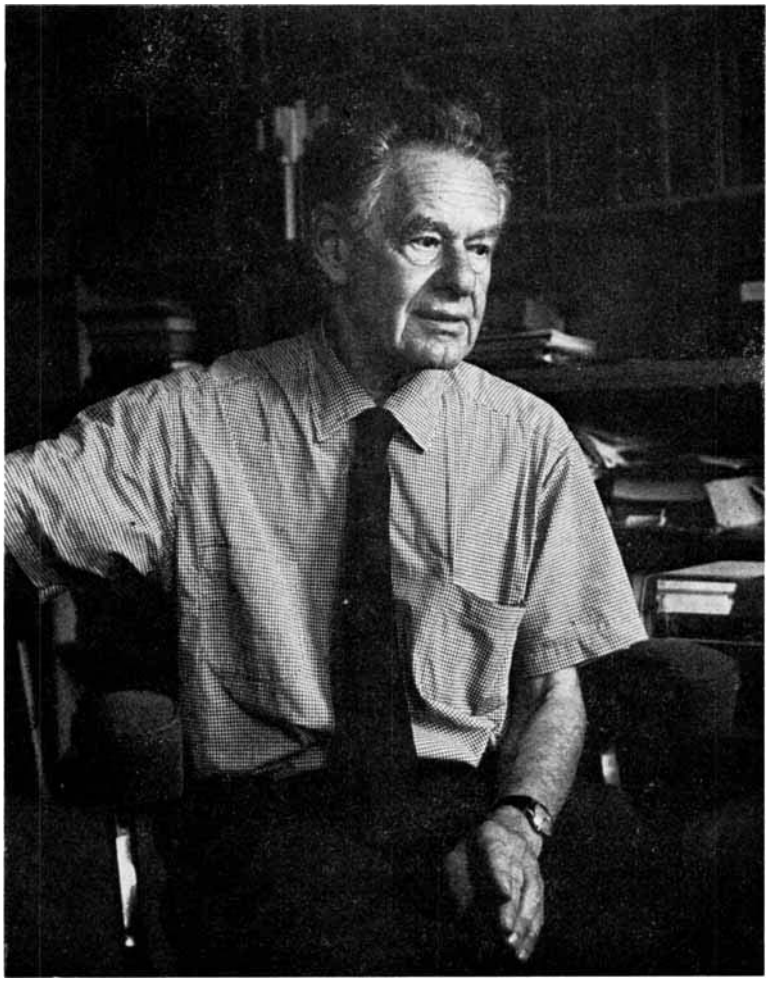

FRITZ LIPMANN

[Photograph by Heka]

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Opening remarks

A. F. HUXLEY

Department of Physiology, University College London

The Ciba Foundation describes its symposia as multidisciplinary international meetings but, since all symposia nowadays tend to be multidisciplinary, we may claim this one to be 'more multidisciplinary than thou'. The objective of such a symposium is cross-fertilization between the distantly related subjects represented here. The analogy of cross-fertilization is drawn from animal breeding and seems to be appropriate for the description of progress in a science. Although cross-fertilization is important for the improvement of, say, a breed of sheep, other analogies may be drawn between scientists and sheep: both have tendencies to follow *en masse* when a new idea gets a head. Darwin pointed out that crossing, though necessary, is a minor matter in improving the breed in the face of the slow *selection* over long periods. This is true for scientific progress; despite the occasional bright idea from outside a discipline, 99% of progress is the steady work of demolishing the many theories that unfortunately turn out to be wrong and of establishing, as far as is possible, facts by experiment—there is no royal road towards progress. In each subject that we shall deal with, the number of possible mechanisms is almost unlimited, but, for example, the great majority of the many mechanisms of muscle contraction that have been put forward independently in the last 20 years have little contact with what is known experimentally.

How far should we speculate and how far should we stick to ideas which are to some degree based on experimental evidence? A pervasive feeling, undoubtedly to some extent justified, is that similar processes are followed in similar ways in different biological contexts. Thus, we hope that an understanding of how energy is transformed from a photon into the making or breaking of a chemical bond may help us to discover how energy is transformed from some chemical event into, say, movement of a muscle. Although we accept partially the uniformity of nature, we do not know the circumstances

in which the uniformity will appear until after the event. Until a problem has been resolved it is possible to make the most terrible mistakes; the history of science is full of such mistakes. For instance, that both smooth muscle and striated muscle contract is a perfectly valid statement but, according to the principle of the uniformity of nature, contraction must then be the same in both cases and so the striations cannot be of essential interest. Such arguments were popular around the turn of the century, before which time the emphasis had been largely on microscopy. They were made explicitly, for example, in the statements of Verworn (1892) and Bernstein (1901), according to whom striations were crude and irrelevant and contraction was clearly something molecular, below the level of what could be seen with the microscope. Attention moved away from the micrometre scale of what was visible with the microscope towards the objective of finding the molecular explanation directly. As it happened, sliding filaments were proposed twenty years ago and showed that this approach had been in a sense wrong. No doubt, however, smooth muscle has essential analogies with striated muscle, but we still do not know just what form these analogies take.

In the contractile field, Weis-Fogh has recently demonstrated a conspicuous example of the non-uniformity of nature. Organelles of *Vorticella* and related protozoa work by a mechanism which 25 years ago was an attractive candidate for the mechanism of muscle contraction. The interest of muscle physiologists in these mechanisms disappeared with the advent of sliding filaments, but the work of Weis-Fogh and others has provided a 'substrate' for these theories which now apply to this structure, the spasmoneme. So, here is a case where, despite the theory originally having been inappropriate for the structure to which it was intended to apply, it has turned out to be applicable in another related area. Clearly, we cannot predict how ideas from one discipline will affect progress in another.

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The roots of bioenergetics

FRITZ LIPMANN

The Rockefeller University, New York

Abstract Understanding metabolic energy transformation began with the realization of an 'intrusion' of phosphate into the mechanism of alcoholic fermentation. The discovery of an analogous participation of phosphate in muscle glycolysis connected the metabolic generation of energy-rich phosphate bonds fed into a common transmitter, adenosine triphosphate (ATP), with the production of mechanical energy through the finding that the phosphoryl group of creatine phosphate transferred to ATP could supply the energy for muscle contraction. In this way, a functional applicability of the energy of the phosphate bond was first shown. This observation was soon followed by the recognition that the phosphoanhydride bond of ATP provided the driving force in biosynthetic reactions; in this type of bond, metabolic energy apparently collects before it is transmitted for functional and biosynthetic use.

The storage of energy in ATP was first detected in anaerobic energy-yielding reactions but soon was also found in respiratory and photosynthetic energy production. However, the mechanism by which energy derived from metabolites was converted into phosphate-bond energy in the latter processes appeared to differ from that of anaerobic energy transmission. Whereas phosphorylated compounds mediate the latter in homogeneous solutions, aerobic phosphorylation and photophosphorylation in prokaryotes seem to require special submembranous structures; and in eukaryotes, energy conversion is a function of special organelles, the mitochondria and chloroplasts.

The evolutionary aspects of the transition from prokaryotes to eukaryotes are of considerable interest. In conclusion, the relevance of an apparent prokaryotic origin of the energy-transforming organelles in the eukaryotes will be commented on.

I shall begin with an eye-witness account of my years in Meyerhof's laboratory, from 1927 until I left in 1930. While there, I was only slowly drawn into the work on bioenergetics; I was observing and learning rather than doing any work of importance myself. Later, I did get into other problems, but eventually when I had leisure to work on my own in the Carlsberg Laboratory in Copen-

hagen (Lipmann 1934*a*, 1935), I drifted back into the exciting developments in the mechanism of fermentation and glycolysis. Subsequently, as I became more and more involved in the mechanism of energy transformation, I graduated from an onlooker to a participant (Lipmann 1939, 1940, 1941). In Meyerhof's laboratory I had the good fortune to live through the time when attention was first turning to the importance of phosphate in intermediary metabolism through the discovery of two key compounds, creatine phosphate (Cr~P; see p. 7) (Eggleton & Eggleton 1926; Fiske & Subbarow 1927) and adenosine 5′-triphosphate (ATP) (Lohmann 1929; Fiske & Subbarow 1929).

I intend to trace the circuitous and slow development of the understanding of metabolic energy transformation through the analysis of the intermediary reactions in cell-free fermentation in yeast extracts and in cell-free glycolysis in muscle extracts. For such an analysis, it was essential to have cell-free extracts available for the bulk conversion of glucose into ethanol and carbon dioxide as well as a conversion of glucose into lactic acid. The first preparation of this kind, a cell-free extract of yeast that promoted vigorous fermentation, was made by the brothers Büchner in 1896. This extract has yielded all the enzymes that catalyse the intermediary steps of metabolic energy conversion, present there mixed in a homogeneous solution. I dare to call this isolation of a solution containing a normally-functioning metabolic system the dawn of what we now call molecular biology.

THE DISCOVERY OF PHOSPHATE AS PARTICIPANT IN FERMENTATION

Harden and his colleagues used this cell-free fermentation to probe into its mechanism. Early in this century, Harden & Young (1906) published on the role of phosphate; they discovered that inorganic phosphate was necessary to promote rapid fermentation and that about one mole of phosphate was esterified for every mole of alcohol and carbon dioxide formed. The product was a hexose diphosphate. In other words, for every pair of (alcohol + CO_2) formed, two phosphates were fixed to the hexose molecule (equation 1).

$$2\text{glucose} + 2\text{P} \rightarrow 2(\text{ethanol} + CO_2) + \text{hexose diphosphate}$$

The primary observation on the participation of phosphate is illustrated in the rate curve (Fig. 1), taken from a study on yeast fermentation in the middle thirties (Lipmann 1934*a*). The curve shows the characteristics of cell-free fermentation; the autocatalytic period of the extract, which contains phosphate and glucose, can be abolished (see later) by the addition of phosphate donors. Once the fast period of fermentation ensues, phosphate is fixed according to the Harden–Young equation. Some glucose 6′-phosphate is formed with the

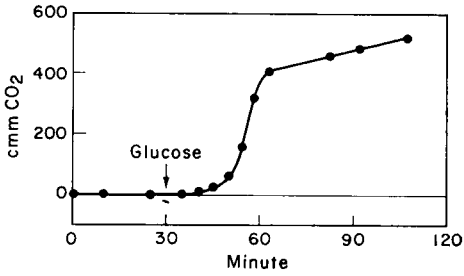

FIG. 1. Manometric determination of CO_2 evolution (in mm^3). The Warburg vessel contained a 10% solution (0.3 ml) of yeast juice dry powder in water, and in the annex a 38% glucose solution (0.05 ml). The arrow indicates the dipping in of glucose. After about 60 min, the free phosphate was used up and there followed a slow fermentation of the fructose diphosphate formed.

hexose diphosphate, which was later identified as fructose 1′,6′-phosphate. When the inorganic phosphate is exhausted (after 60 min in Fig. 1), fermentation slows dramatically. Then, large amounts of fructose diphosphate are present but are fermented slowly. However, when new inorganic phosphate is added (not shown) fast fermentation starts again. The puzzle posed by the need for inorganic phosphate in fermentation, as well as the relative inactivity of the hexose diphosphate, even though it impressed as being an intermediary in alcoholic fermentation, had to wait for almost 30 years to be fully resolved.

THE PARALLEL BETWEEN FERMENTATION AND GLYCOLYSIS

When I arrived at Meyerhof's laboratory, glycolysis was being studied with a muscle extract which was prepared by the extraction of rabbit muscle with distilled water (Meyerhof 1927); it contained practically no glycogen and only poorly glycolysed free glucose. In order to obtain a glucose metabolism analogous to that in yeast extracts, Meyerhof prepared from yeast autolysates a fraction which he called glucose activator and later found to be hexokinase (Meyerhof 1930) that was virtually absent from these muscle extracts. With the combination of muscle extract and hexokinase, he obtained exact parallels between glycolysis and fermentation with regard to fast rate in the presence of an excess of inorganic phosphate and its fixation as fructose diphosphate: in other words, the glycolytic equivalent of a Harden–Young reaction with lactic acid as product rather than ethanol and carbon dioxide.

The progress of the combined studies on alcoholic fermentation and muscle glycolysis seemed to justify the expectation that one might open the 'black box' wherein one hoped to find the then mysterious transformation that would

yield out of the initial substrate, glucose, the end products of fermentation, $C_2H_5{\cdot}OH + CO_2$, and of glycolysis, $CH_3{\cdot}CH(OH){\cdot}COOH$ (lactic acid).

THE LINK OF GLYCOLYSIS TO MUSCLE CONTRACTION

Meyerhof proposed that lactic acid production was directly connected with muscle contraction (Meyerhof 1925). He reached this conclusion by finding "... as a general rule that a fixed relation exists under normal conditions between the lactic acid formed (anaerobically) upon stimulus, and the developed isometric tension...". This he called the isometric coefficient of lactic acid and concluded that it indicated that the production of lactic acid is mechanistically connected with mechanical response. Meyerhof was aware that, in order to understand the physiological significance of metabolic reactions, it was essential to know, in addition to its chemical equation, the change of energy during the reaction. Calorimetry was used for such estimates (Meyerhof & Lohmann 1928). Thus, when two new phosphate-containing compounds, Cr$\sim$P and ATP, were discovered in muscle, although far from realizing their bioenergetic importance, Meyerhof & Lohmann determined their heat of hydrolysis. They found it to be surprisingly high, about 50 kJ/mol Cr$\sim$P (12 kcal/mol) and about 50 kJ/mol for each of the two terminal $\sim$P in ATP. In 1927, the Eggletons, in a short note on the significance of phosphate in contraction (Eggleton & Eggleton 1927), reported briefly some data which they rightly interpreted to mean a connection between breakdown of creatine phosphate (they called it phosphagen) and muscular action. However, in view of the convincing link established with lactic acid formation, such a connection was not seriously considered in Meyerhof's laboratory when I joined it in 1927 (cf. Meyerhof & Nachmansohn 1928).

A MUSCLE CONTRACTION CAUSED BY BREAKDOWN OF CREATINE PHOSPHATE

Lundsgaard (1932) studied the contraction of iodoacetate-poisoned muscle from rats. Muscles which had contracted for a brief period were entirely free of the lactic acid normally formed (Fletcher & Hopkins 1907; Meyerhof 1925). In view of this, Lundsgaard tested for breakdown of Cr$\sim$P and found that it closely paralleled tension; after exhaustion of the small store of creatine-bound phosphate, the muscle went into rigor.

In the light of these results, the significance of Meyerhof's and Lohmann's finding (1928) of the unusually large heat obtained by hydrolysis of the N–P bond in Cr$\sim$P became obvious. Lundsgaard called the contraction of an

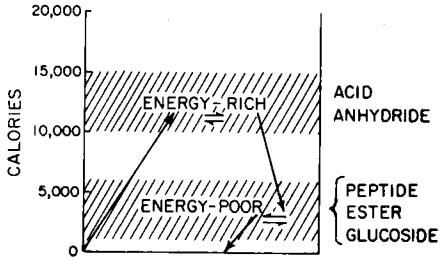

FIG. 2. Scheme of prevalent levels of free energy (ΔG) of hydrolysis (modified from Lipmann 1950). The energy-rich level represents metabolites of the acid anhydride type, such as pyrophosphates, carboxy- and enol-phosphates, thioesters and glycol-amino esters as in aminoacyl-tRNA, in contrast to the energy-poor type of compound shown on the right. (The sharp demarkation between the two levels is exaggerated. There appears to be a more gradual transition between energy-rich and energy-poor free energies than indicated in this scheme.) The upward arrow marks the direction of metabolic generation of energy-rich bonds. The downward arrows mark use in biosynthesis with eventual liberation of the energy-carrying annex to what is designated here as zero level, as for example in inorganic phosphate or CoASH to re-enter the metabolic cycle by way of the upward arrow.

iodoacetate-poisoned muscle an alactacid contraction, after proving that its glycolysis was completely blocked. This work created a great disturbance in Meyerhof's laboratory, shattering the confidence in a direct connection of lactic acid to the mechanical work done. The rationalization of the relation between breakdown of Cr~P and contraction was greatly furthered when Lohmann (1934) connected Cr~P with phosphorylation of ADP by showing in muscle extract that ADP acted as a cofactor since, after dialysis, Cr~P was not split in the absence of ADP to which its ~P was transferred to yield ATP. In other words, phosphoryl transfer was catalysed by the intermediate acceptor ADP. This feature was to become the prototype of a general event in intermediary metabolism, namely, that metabolically generated energy-rich (Lipmann 1941) phosphate bonds in nearly all cases were converted into ATP for metabolic use. I introduced the wiggle sign (~) to distinguish energy-rich or high-energy phosphate bonds with a free energy of hydrolysis of around and above 42 kJ (10 kcal) from the energy-poor bonds in ordinary phosphate esters. It marked the ~P derivatives as energy-carriers, on account of what I began to call their high phosphoryl potential. The metabolic cycling I proposed from inorganic-P to ~P through energy utilization back to inorganic-P, generalizing to other energy-carrying combinations, is illustrated in Fig. 2.

GYCOLYSIS IDENTIFIED AS ATP DONOR TO MUSCLE

To return to the slowly developing identification of the intermediate steps in

glycolysis and fermentation, one now began to realize that glycolytically derived ATP—but not lactic acid—is used to drive the muscle normally, and Meyerhof's results, thus re-interpreted, proved correct: they now implicated the delivery of ATP with the production of mechanical energy. In the meantime, the sluggishness of glycolysis and fermentation of hexose diphosphate in extracts metabolizing glucose rapidly at optimal conditions had raised doubts about the insertion of phosphate in fermentation and glycolysis. This was chemically uncomfortable since so little was known then about organic phosphates. It was seriously proposed that the formation of hexose diphosphate was an unphysiological side reaction. The road to its true appreciation was paved by two observations on the reactivity of fructose diphosphate. Nilsson (1930) found with dry yeast that in the presence of fluoride ions (a fermentation inhibitor) fructose diphosphate reduced acetaldehyde rather easily to ethanol and, as the equivalent oxidation product, he found, to everybody's surprise, phosphoglyceric acid, which appeared for the first time in a fermentation experiment. This acid was well characterized by crystallization as the barium salt.

It is surprising now that nobody—including myself—seemed to connect this with the early realization that at some stage in the course of fermentation and glycolysis the hexose molecule had to be split into two C_3 compounds to yield $CH_3 \cdot CH(OH) \cdot COOH$ or $C_2H_5 \cdot OH + CO_2$. After the three-carbon phosphoglyceric acid appeared on the horizon as intermediary, it still took quite a while to associate this oxidation of fructose diphosphate with the oxidation of a triose phosphate.

The second observation that led eventually to the understanding of the oxidoreductive events was made by Lohmann (1930). He found that muscle extract, in the presence of fluoride ions, converted the ester phosphate in fructose diphosphate into a form which was more difficult to hydrolyse with acid. He asked me to help him find out if fluoride was necessary (Lipmann &

TABLE 1

Conversion of fructose diphosphate into an acid-stable phosphate ester in muscle extract of winter frogs after incubation at 20 °C

Incubation	*Phosphate bound (mg P_i)*	*Phosphate hydrolysed by acid in 3 h (mg P_i)*	*Converted phosphate (mg P_i) (%)*
0	0.48	0.48	
20	0.50	0.39	0.12 (25)
60	0.50	0.25	0.27 (53)
120	0.49	0.19	0.33 (68)

From Lipmann & Lohmann (1930).

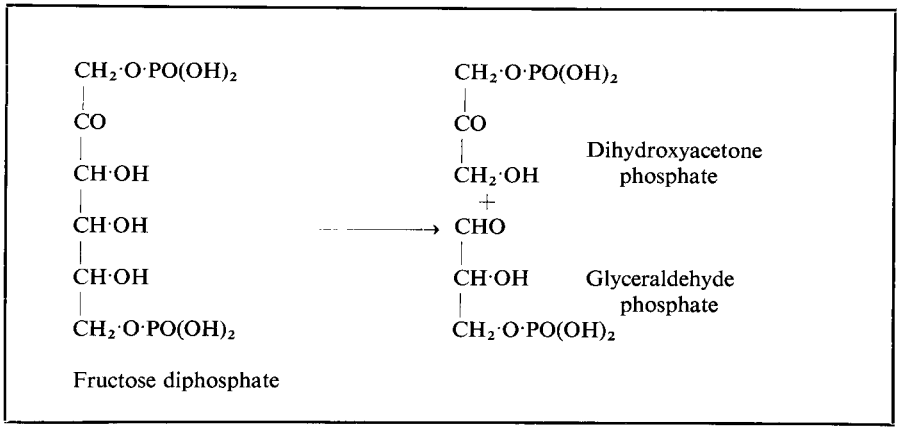

FIG. 3. Aldolase split of fructose diphosphate in muscle preparations (after Embden *et al.* 1933).

Lohmann 1930). If not, the reaction would be more likely physiological. I found with some muscle extracts that essentially the same conversion took place without fluoride ions. Such a conversion is illustrated by Table 1.

The reactivity of fructose diphosphate in muscle extract prompted Embden *et al.* (1933) to similarly incubate the diphosphate with muscle preparations, but they then proceeded to analyse more thoroughly for the compounds formed. They discovered that fructose diphosphate was not converted into a hexose ester which was difficult to hydrolyse, as presumed, but, rather, was broken down to C_3 compounds which were difficult to hydrolyse, since they found phosphoglyceric acid and some phosphoglycerol as products. With Nilsson's results in mind, they then concluded that this could only mean a triose phosphate split followed by a dismutation.

Such a conclusion really was indicated, the more clearly so from Nilsson's experiments where fructose diphosphate was oxidized by acetaldehyde to the well characterized phosphoglyceric acid. But the connection was not made. Only Embden saw the light: without actually having the full chemical evidence, he concluded that such a triose split of the diphosphate would yield glyceraldehyde phosphate (Fig. 3) and dihydroxyacetone phosphate, and that these two would interact to yield glycerol phosphate, with the glyceraldehyde phosphate being oxidized to phosphoglyceric acid (Fig. 4). His observation suggested in one sweep many of the intermediary steps that had to be postulated for glycolysis and fermentation. However, at that stage, the explanation reached only the level of an oxidation–reduction (and the dismutation was really not on the main path of glycolysis); it did not extend to the energy transformation being the true implication of the insertion of phosphate. This, however, soon developed.

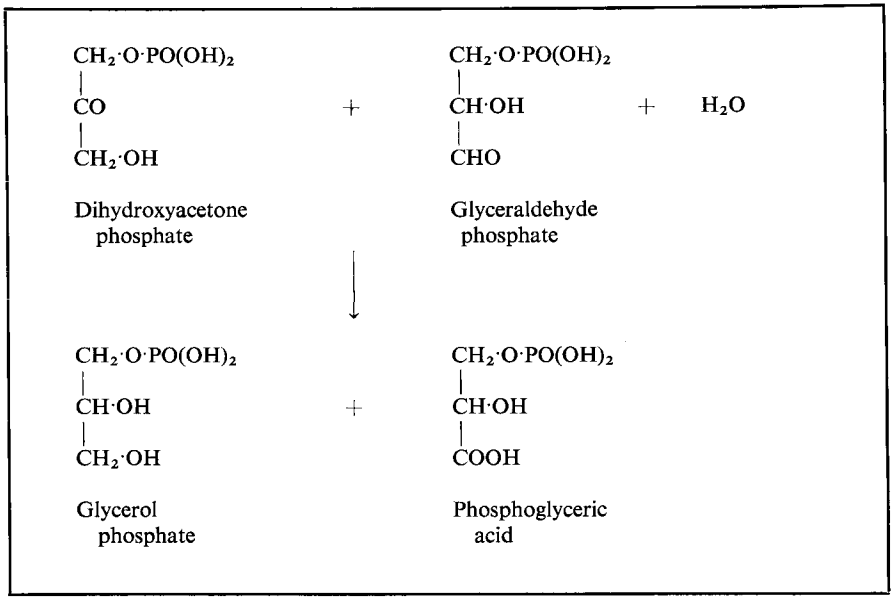

FIG. 4. Dismutation in the metabolism of fructose diphosphate (see Fig. 3) in muscle preparations (after Embden *et al.* 1933).

It had been observed in Embden's experiments that phosphoglyceric acid was readily split into pyruvate and inorganic phosphate in muscle extracts (Fig. 5). Furthermore, other experiments (Parnas *et al.* 1934) suggested that phosphoglyceric acid yielded ATP on incubation in muscle preparations. These results began to indicate that in some manner the energy-poor phosphate ester in phosphoglyceric acid was converted into an energy-rich form. Some

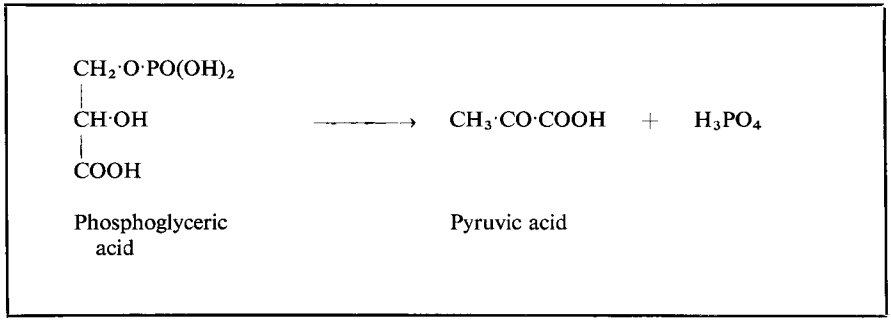

FIG. 5. Formation of pyruvic acid from phosphoglyceric acid (see Fig. 4) (after Embden *et al.* 1933).

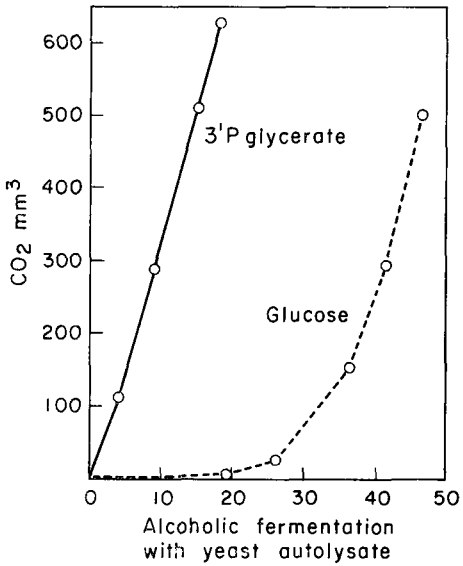

FIG. 6. Abolition of the induction period in glucose fermentation in yeast extract by 3′-phosphoglycerate. Alcoholic fermentation with yeast autolysate (in min) was measured as described in Fig. 1. When 0.1 ml of 30% glucose alone (----) was added, a long induction period led over an autocatalyic period to fast phosphate-linked fermentation. With 0.2 ml of 0.016M-phosphoglyceric acid added and glucose (——), fast fermentation began immediately. In both cases, substrate was dipped in at zero time.

of my own observations convinced me of the truth of such an assumption. Addition of phosphoglyceric acid with glucose to yeast autolysate eliminated the induction period (see Fig. 6; Lipmann 1934*b*). Such an effect, as mentioned earlier, indicated the facilitation of phosphate transfer to glucose. The phosphoglyceric acid eliminated the sluggishness with which the energy-rich phosphate was 'found' to prime glucose by conversion into glucose phosphate. Because phosphoglyceric acid was one of the best inducers that I could find, its conversion into a ~P-containing compound donating phosphate to glucose via ATP and hexokinase was strongly indicated. This was elaborated in the work of Lohmann & Meyerhof (1934), who analysed the chain of reactions leading from 3′-phosphoglyceric acid to a ~phosphoryl group, transferable to ADP; the first step was conversion of 3′- into 2′-phosphoglyceric acid, then by dehydration into energy-rich phosphoanhydride-like phospho~enolpyruvate (cf. Lipmann 1941) and thence to ATP by transferase to ADP.

$$HO{\cdot}CH_2{\cdot}CH(O{\cdot}PO_3^{2-}){\cdot}COOH \xrightarrow{-H_2O} CH_2{:}CO(\sim PO_3^{2-}){\cdot}COOH$$

Again, the metabolically available ~P was fed into the ATP pool.

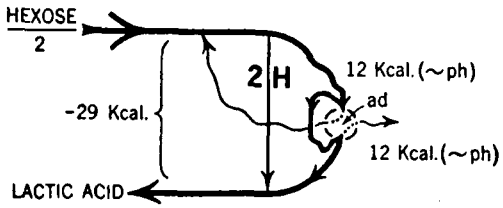

FIG. 7. The flow sheet of glycolysis. The internal consumption of one ATP for phosphorylation of half a hexose is indicated by the upward arrow. Therefore, the net yield from the two ~P yielded by fermentation of a triose half is only one ATP, indicated by the arrow to the outside.

There still remained the second part of the puzzle: why is inorganic phosphate needed? This was solved by Warburg & Christian (1939): the inorganic phosphate was essential in the oxidation of glyceraldehyde 3′-phosphate where it was fixed as an energy-rich carboxy phosphate anhydride in 3′-phosphoglyceryl~phosphate. Thereby, the energy of oxidation was saved in the ~P link to the carboxy group. This ~P was transferred to ADP and thereby another type of ~P was shown to be generated in the course of glycolysis and fermentation.

Thus, by 1935–1936, all the intermediary steps in glycolysis had been established, and it became evident that the phosphate acted in the mechanism of fermentation and glycolysis as the energy carrier in the form of energy-rich phosphoryls developed in the metabolic transformations. As shown in Fig. 7, for each half glucose, one phosphoglyceryl~phosphate and one phospho~enolpyruvate are generated and collected in two ATPs. Therefore, for every mole of glucose, four ATPs are yielded; two are returned into the reaction to form fructose diphosphate and two are the net yield of glycolysis. So, here we have the answer to the question of why glycolysis can drive the muscle machine that depends on the transfer of energy-rich phosphate to myosin (Engelhardt & Ljubimova 1939; Needham *et al.* 1941; Szent-Györgyi 1941–1942). Obviously, all the energy that can be derived from glycolysis flows into energy-rich phosphate bonds to be delivered into the ATP pool (Fig. 7). In the absence or insufficiency of metabolically formed ~P, as for instance when glycolysis is inhibited, the ~P in Cr~P, by producing ATP, as ~P buffer causes contraction (cf. Eggleton & Eggleton 1927).

USE OF ATP IN BIOSYNTHESIS

Some of my own observations helped to generalize on the function of the phosphoryl in ATP as a donor not only in the production of mechanical energy

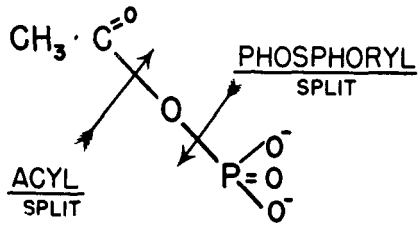

FIG. 8. The double function of acetyl phosphate; (i) acetyl donor to CoA with phosphate acetyltransferase and (ii) phosphoryl donor to ADP with acetate kinase.

but also in biosynthesis. During work on the oxidation of pyruvate with extracts of *Lactobacillus delbrueckii* (Lipmann 1939), I accidentally observed that oxidation of pyruvic acid in this cell-free system required the presence of inorganic phosphate. Just at that time Warburg had discovered that the role of inorganic phosphate in alcoholic fermentation is, so to say, to preserve redox energy by transfer of the activated carboxy group to form an energy-rich phosphate. With oxidation of pyruvate to acetate and CO_2, the phosphate again prevents energy dissipation by connecting phosphate to the activated carboxy group of acetate. In this case, the acetyl phosphate seemed to be a candidate for the 'active' acetate postulated by Schoenheimer (1942) from his work with [^{2}H]acetate. [^{2}H]Acetate had been shown to be a precursor of many essential components of the living organism, its deuterium atom appearing in cholesterol, steroids, fatty acids and many amino acids. The production of an activated acetate in the form of acetyl phosphate made one argue that the acetyl phosphate could not only act as a phosphoryl donor to ADP but also as an acetyl donor (Fig. 8). Subsequently, this acetyl was found not to be donated directly but after intermediate transfer to a specific acetyl carrier, coenzyme A. Stadtman & Barker (1950) had discovered in bacteria an enzyme that exchanged inorganic phosphate with acetyl phosphate which they called transacetylase and which was found to be CoA-dependent (Stadtman *et al.* 1951). This made us conclude that the enzyme reversibly transferred the acetyl group from phosphate to CoA. An acetyltransferase activity of mammalian phosphoglyceraldehyde dehydrogenase reversibly connecting acetyl phosphate and CoA was found by Harting & Chance (1953). Its physiological significance, however, was considered problematical.

Thus, a metabolite that was linked in an energy-rich bond to phosphate was shown to be primed for transfer to CoA from which it catalysed condensation to fatty acids and steroids. This showed for the first time that ATP could promote biosynthetic reactions by way of a compound like acetyl phosphate.

A host of biosynthetic pathways were soon discovered in which the mere addition of ATP to extracts in combination with the ingredients for a condensation initiated that condensation, so that the generalization seemed to be warranted that one can consider the phosphoryl of ATP as a general energy donor. This is also to be concluded from the ability of a yeast to grow anaerobically with ATP-producing fermentation as the only source of energy.

It cannot be considered an accident that the sequence of reactions leading, in metabolic energy generation, to the formation of energy-rich phosphate bonds, was discovered in anaerobic fermentations. These could be analysed in homogeneous solutions where the enzymes responsible for the intermediate steps could all be separated and the initial phosphoryl carriers donating phosphate to ATP could be identified.

AEROBIC PHOSPHORYLATION AND PHOTOPHOSPHORYLATION

For a short while, it was argued that perhaps this manner of energy transformation occurred only in anaerobic metabolism, but soon phosphorylations connected with the respiratory chain were found in kidney homogenates by Kalckar (1938) and in muscle by Belitser & Tsybakova (1939). In addition, a photophosphorylation was found in bacterial extracts (Frenkel 1954) and in chloroplasts (Arnon *et al.* 1954); it became clear, furthermore, that in photosynthesis the synthesis of carbohydrate from CO_2 was coupled with phosphorylation of intermediates. Aerobic phosphorylation occurs in submembranous lamellae of bacteria and in mitochondria (Lehninger 1964), presumably originating from bacterial symbionts (Stanier 1970). Photophosphorylation is observed in photosynthetic bacteria and in blue-green algae in submembranous lamellae, and in plants in chloroplast-organelles presumably originating from symbiont blue-green algae (Stanier 1970).

The reactions will not proceed in homogeneous solutions; they require the intervention of the membranes. After a long and seemingly unsuccessful search for phosphorylated intermediates in aerobic and photophosphorylations, Mitchell (1961) proposed a mechanism quite different from that in anaerobic 'extract' phosphorylation in that protons and hydroxy ions are separated, creating a membrane potential. In this way, the theory of respiration proposed by Lundegardh (1945) (see Fig. 9) was revived. Mitchell presented preliminary evidence that a membrane potential thus set up might be converted into phosphate bond energy by a dehydration between ADP and inorganic phosphate that was catalysed by the ATPase connected with these membrane systems (Fig. 10) (Fernandez-Moran *et al.* 1964). Although it is taking some time for the Mitchell interpretation to be generally accepted, it appealed to many from

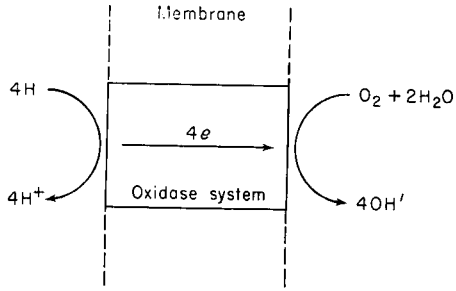

FIG. 9. Charge separation by respiration.

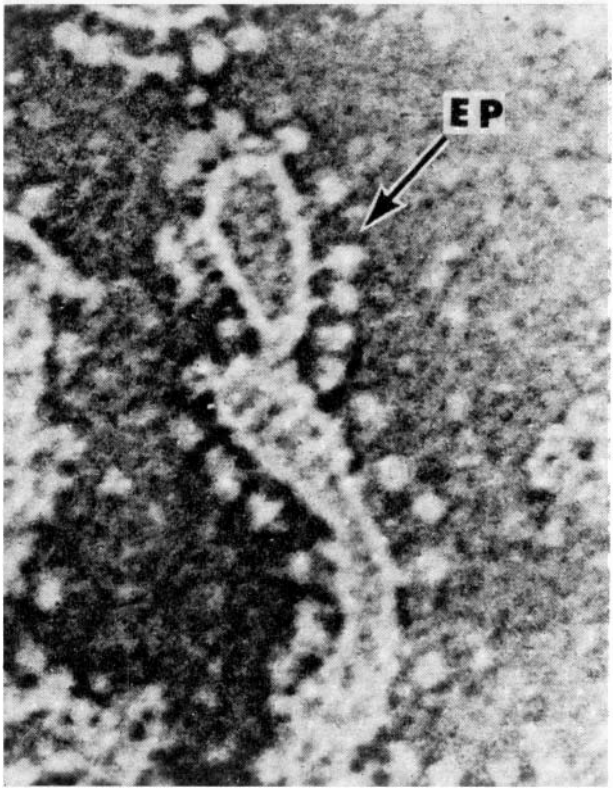

FIG. 10. The membrane-linked, ATPase-containing globules seen in the electron micrograph of mitochondria.

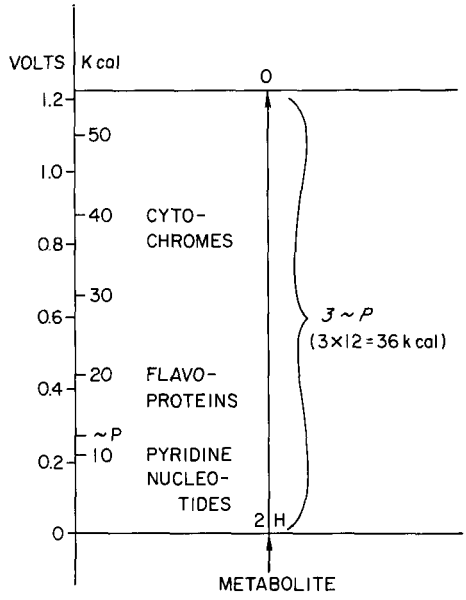

FIG. 11. Conversion of electron flux energy into ~P. The bracketing of the formation of three ~P (36 kcal ≈ 150 kJ) by the transfer of substrate hydrogen to oxygen through several redox levels indicates that it occurs by a uniform mechanism of ~P transfer to ADP. This is probably an oversimplification.

the beginning as a welcome way of explaining the absence of phosphorylated intermediates in aerobic phosphorylation. Influenced by the all-or-nothing effect of uncouplers such as dinitrophenol (Loomis & Lipmann 1948) on respiration and phosphorylation, this indicated a common mechanism at different redox levels and for quite a while I have come to indicate this in lectures by a scheme for aerobic phosphorylation represented in Fig. 11. There now appear to be good indications that the steps generally assigned to the three phosphorylation steps in the transfer of electrons do produce ATP instead by a common mechanism.

In the type of ATPase attached to the ends of myosin threads in Fig. 12 (Slayter & Lowey 1967) I like to see some similarity to the ATPase attached by a thread to the mitochondrial membrane that catalyses aerobic phosphorylation and I wonder if a new look at the energy transfer from ATP to myosin might not uncover parallels between it and the mechanism of aerobic phosphorylation.

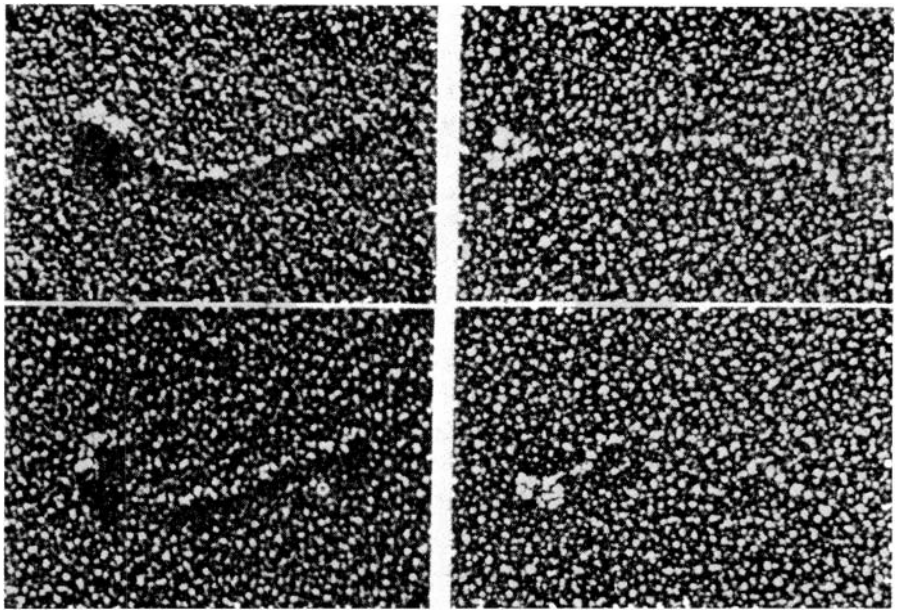

FIG. 12. Electron micrograph of heavy meromyosin: the two ATPase globules at the end of the double-stranded meromyosin are histologically similar to the ATPase globules on the mitochondrial membrane in Fig. 10. Both ATPases catalyse energy transformation.

EVOLUTIONARY ASPECTS

A generally accepted dogma says that the early Earth had a reducing atmosphere. The early prokaryotic (i.e. bacterial) organisms had to rely, therefore, on an anaerobic energy supply, and many present-day bacteria still do. More recently, when atmospheric oxygen had become available, prokaryotes developed the more economical respiration, and eventually transition to eukaryotes took place (Stanier 1970; Gel'man *et al.* 1967). One outstanding difference between these two basic lines of organisms lies in the fact that the eukaryotes possess organelles in which the main energy transducing systems are located: the mitochondria for respiration and the chloroplasts for photosynthesis. These mitochondria and chloroplasts appear to have originated from prokaryotes symbiotically associated with a 'host' cell. The prokaryotic origin presumably as symbiont was first strongly suggested by the finding of a DNA in organelles (Ris & Plaut 1962; Nass & Nass 1963; Nass 1969) that was different from nuclear DNA in composition and that was, as in prokaryotes, of a circular nature.

The presumably prokaryotic origin of the mitochondria came to my attention when, in the course of extensive work on prokaryotic and eukaryotic ribosomal protein synthesis (Lipmann 1969*a*; Lucas-Lenard & Lipmann 1971), we investigated the type of protein synthesis peculiar to mitochondria (Krisko *et al.* 1969; Richter & Lipmann 1970; Richter 1971) and also chloroplasts (Sy *et al.* 1974). We found then that the complements to ribosomes, the so-called supernatant factors in mitochondrial protein synthesis, were prokaryotic, whereas the cytoplasmic ribosomes, which are more abundant in the eukaryotic cell, were complemented by a set of truly eukaryotic factors (Richter & Lipmann 1970).

The most interesting aspect of this is our ignorance about the origin of the receiving part, the host for these prokaryotic organelles (Margulis 1971). It is significant that the prokaryotes remained single-celled and seemed to be unable to evolve in the sense we tend to use the word 'evolution'. Rather, they were 'inventive' and created an amazing metabolic diversification. They prepared evolution by the synthesis of nucleotides, amino acids and coenzymes, and, most importantly, they constructed the chain for transfer of genetic information, DNA $\rightarrow$RNA $\rightarrow$protein, creating for the last step the universal genetic code. The prokaryotes possess an enormous potential to develop different metabolic pathways, including anaerobic use of all kinds of inorganic compounds such as nitrate and sulphate as hydrogen acceptors and molecular hydrogen as donor, systems which, among many other metabolic features, the eukaryotes have discarded, thereby simplifying their metabolic repertoire.

One of the most drastic metabolic simplifications was eventually adopted by the higher organisms when they abandoned their own manufacture of eight of the twenty amino acids (Fruton & Simmonds 1958) and of nearly all the complex metabolic accessories we call vitamins (Lipmann 1969*b*). To concentrate on progressive evolution, eukaryotes unburdened themselves by importing these essentials and, furthermore, by adopting a rather standard energy metabolism. This eased the way for the orientation towards, adaptation to, and eventual domination of the environment. Part of the advantage gained by eukaryotes must be sought in their enclosure of energy transduction into organelles. Thereby, the energy-generating system was taken out of the cell proper and appropriately prepared energy carriers were delivered to it for use, just as in daily life we plug into dispensers of appropriately transformed energy.

We can draw a speculative plot of 'evolution' against the age of the Earth, assuming it to be about 5000 million years old (Fig. 13). The first prokaryotes probably appeared about 3500 million years ago; then came the metabolic plateau from which evolution was to take off. It took about 2000 million years

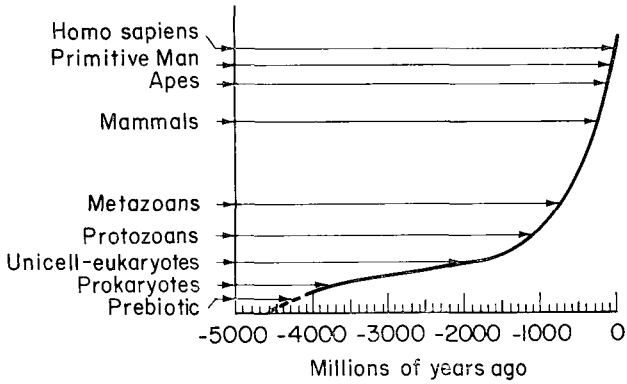

FIG. 13. Speculative plot of evolution against the age of the Earth.

before the appearance of the unicellular eukaryotes which one has to consider as the real ancestors of higher organisms (Schopf 1970). Then the rate accelerated; multicellular organisms developed with increasing complexity, culminating in the creation of man. The 'break' between prokaryotes and eukaryotes thus happened about 2000 million years ago. The creation of the compounded eukaryotic cell appears to have resulted from the investment of prokaryotic symbionts, including the precursors of the energy-transducing chloroplasts and mitochondria. Many, including myself, believe that the chloroplasts of plants originated by investment of the highly developed but non-nucleated blue-green algae that everybody agrees to classify with the prokaryotes.

The prokaryotic origin of mitochondria is more obscure because they became more integrated with the host cell (Richter 1971). Their DNA is much smaller than that of the chloroplasts. Most of the respiratory enzymes and catalysts are synthesized in the cytoplasm on messenger RNAs derived from nuclear DNA. The number of messenger RNAs derived from the small mitochondrial DNA consists of a few enzymes, some tRNAs, and 'structural' proteins (Nass 1969) that connect respiratory catalysts such as cytochrome *a* with internal convoluted membranes of the mitochondria.

The logarithmic increase in rate through mammals and apes culminated in a strong evolutionary pressure for brain development in the creation of the species *Homo sapiens* (Fig. 13). To find the reasons for this recent increase in the rate of evolution seems to me a most interesting problem.

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For the preparation of this paper, Herman Kalckar's excellent selection of, and annotations to, pertinent papers in his *Biological Phosphorylations* (Prentice Hall, 1969) has been most helpful, and should be consulted for additional references.

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For discussion, see pp. 63-68

Energy transfer in mitochondrial synthesis of ATP; a survey

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Abstract The energy transduction in mitochondria, with its principal agent ATP, still represents a major challenge for biological research. In general, the energy transduction process is divided into three sections: (1) the redox processes; (2) a conservation of intermediary energy forms; (3) synthesis of ATP. All three processes are linked to the membrane and are, therefore, as difficult to resolve as are processes linked to other biomembranes.

It is probable that the electron transport system is constructed in such a way as to provide energy for synthesis of ATP and related processes. Important for this function is the transversal distribution of these components across the membrane, facilitating generation of membrane potential by electron or proton transfer. The exact composition of the respiratory chain is not yet known, in particular with respect to iron–sulphur proteins. Progress is achieved by defining single species of the respiratory chain, subunit composition, amino acid sequences and genetic derivation from intra- or extra-mitochondrial translation.

Energy generated by oxidation can be trapped before ATP is formed by a number of reactions, in particular reversed electron transport, energy-dependent transhydrogenation and uptake of anions or cations into the mitochondria. The latter reaction is of major importance for understanding the intermediate energy form, as it appears to use energy most directly and be driven mainly by membrane potential or proton gradient across the membrane.

The formation of ATP is a major problem hindering elucidation of the mechanism of oxidative phosphorylation. The mechanism of this enzymic process is not yet understood although the enzymes have been isolated and the subunits have been defined. Most probably, a concerted reaction between ADP and phosphate, driven by some conformational transition of the complex, leads to the formation of ATP. Release of ATP from a hydrophobic to hydrophilic environment may consume most of the energy.

This paper is meant to give a brief introduction to our current knowledge of the interconversion of respiratory energy into phosphate bond energy during oxidative phosphorylation in mitochondria. I shall summarize some facts and

models for the mechanisms underlying this important energy transduction process. Such a contribution appeared to be desirable in this symposium as no other paper deals with this major synthetic source of ATP in aerobically living eukaryotic cells. I am aware that this treatment cannot be complete and apologize for any omissions or one-sided views presented here.

Energy transformation linked to oxidative phosphorylation in mitochondria has been subject to intensive discussion and controversies over the years. Sometimes, exaggerated ideas have been propounded without sufficient experimental basis. We must realize that the mitochondrial energy transformations are linked to membranes and that, therefore, the level of understanding is part of a general lack of comprehension of the functional processes in membranes. In other words, the research on oxidative phosphorylation does not lag behind research on other membrane-linked functions such as on membrane-bound enzymes or on membrane carriers. On the contrary, the amount of experimental data available on the mitochondrial membrane far exceeds that for any other membrane.

THE SYSTEM OF OXIDATIVE PHOSPHORYLATION

The components comprising the system of oxidative phosphorylation are largely known now. They may be divided into (*a*) the electron transfer components including dehydrogenases and the coenzymes, and (*b*) the ATP synthase complex. Both parts of the system are more or less tightly associated with the inner mitochondrial membrane and, therefore, the isolation and definition of the various protein constituents encounter great difficulties. In particular, it is not possible to isolate the components, as with other membrane-bound enzymes, in the fully active state. Attempts at reconstitution are often unsatisfactory in that they either start from partially purified systems or that the reconstitutive activity is only low as compared to the native system.

The respiratory chain

According to one view, the construction of the respiratory chain is understandable only in terms of its role of providing energy for ATP synthesis. Most of the components are bound to the inner mitochondrial membrane. The approximate occupation density and resulting distribution of the components on the inner mitochondrial membrane is shown in Fig. 1, based on the content of the components and membrane surface in beef heart mitochondria (Kröger & Klingenberg 1970; cf. Klingenberg 1968).

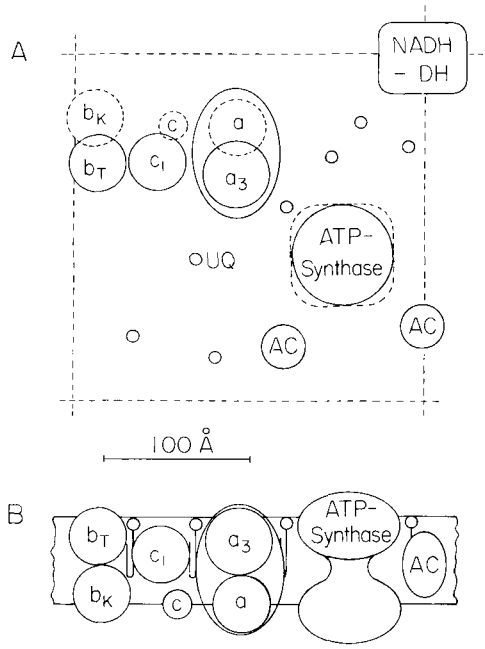

FIG. 1. The possible spatial arrangement of electron-transport carriers and ATP-synthesizing components on the mitochondrial membrane (Kröger & Klingenberg 1970). A, Density of occupation on the membrane; B, distribution across the membrane (100 Å = 10 nm). The density of occupation is calculated from the membrane surface and the content of the component in beef heart mitochondria. AC = carrier of ADP and ATP; UQ, ubiquinone.

As in all biomembranes an asymmetric localization of the membrane-bound components can be expected. It had previously been suggested that cytochrome a_3 is located more towards the inside (Palmieri & Klingenberg 1967) and cytochrome *c* to the outside (Klingenberg & von Jagow 1970; Racker 1970). This arrangement was substantiated by several methods, in particular antibody studies (Racker 1970). The distribution is of great interest with respect to the energy transduction. In addition to the internal NADH dehydrogenase present in all mitochondria a NADH dehydrogenase was found to be localized on the outer surface in mitochondria from fungi and plants (von Jagow & Klingenberg 1970). Note that NADH oxidized by the external pathway yields less energy (P/O = 2) than by the internal enzyme (P/O = 3) (Ohnishi *et al.* 1960).

Most components have been isolated and enzymically characterized. Cytochrome a,a_3 consists of several subunits, the functions of which are not yet known (Tzagaloff *et al.* 1973). Particularly tightly bound to the membrane is

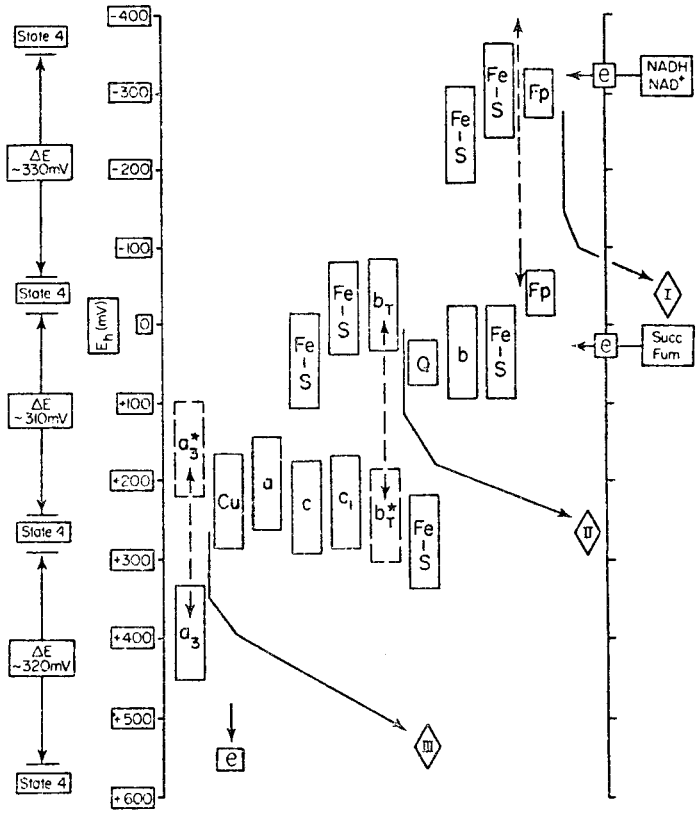

FIG. 2. The redox potentials of the electron carriers of the respiratory chain (from Wilson *et al.* 1973). The rectangles are centered on the half reduction potential at pH 7.2 and extend to the redox potentials at which the component is reduced from 9% to 91%. The three coupling sites are denominated by I, II and III.

cytochrome *b*. Spectroscopically, two different *b*-type cytochromes are seen in animal mitochondria corresponding to cytochrome 'b_T' and 'b_K' (cf. Wikström 1973).

The sequence of respiratory carriers follows largely their redox potentials which span the region between NADH (–320 mV) and oxygen (+800 mV) (cf. Wilson *et al.* 1973) (see Fig. 2). There appear to be three energy-dependent shifts in the redox potential sequence which are attributed to the energy-transfer steps. This apparent shift of the redox potential has been suggested to be an energy-linked reversed electron transfer rather than real (Bonner *et al.* 1974).

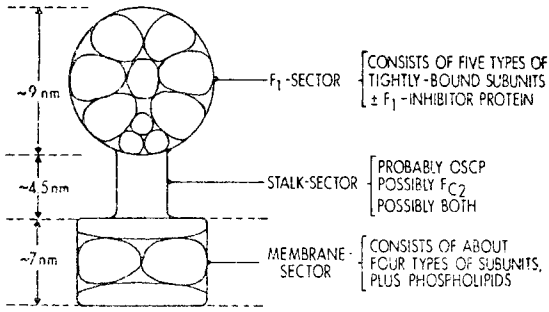

FIG. 3. The subunit structure of the mitochondrial ATP synthase complex (from Senior 1973). The picture describes a tentative speculative subunit structure based on the electron-microscopic evidence for the construction out of a headpiece, a stalk and a base piece of the ATP synthase complex.

The ATP synthase complex

More and more, the protein components associated with the ATP synthase are being characterized. From the whole complex in the membrane called the 'oligomycin-sensitive ATPase', a soluble ATPase can be isolated, which has been called soluble ATPase or F_1, factor A, etc. (cf. Senior 1973; Beechey & Cattell 1973). The notation F_1 appears no longer practical, since the other coupling factors F_2 to F_6 did not survive the progress in further refining the ATP synthase system. The soluble ATPase complex contains the catalytic sites for ATP formation. It consists of six different subunits, the different functions of which are not yet known. More tightly bound to the membrane and probably directly associated with the soluble ATPase is a complex of more hydrophobic proteins which are more difficult to purify, particularly in a functioning state. This section appears to be important in the transfer of energy to the catalytic ATP-synthesizing section. A scheme depicting a hypothetical arrangement of these subunits is shown in Fig. 3. It is based on the electron-microscopically defined 'ATPase-knobs' which protrude from the inner face of the membrane (see Fig. 1) on hypotonic shock treatment.

The coupling sites at the respiratory chain

It has been generally acknowledged for some time that the total energy released by oxidation of NADH is divided into three parts each providing energy for synthesis of one mole of ATP for one mole of NADH oxidized or for one mole of $O_2/2$ taken up ('P/O' = 3). Electrons entering at the level of ubiquinone or at cytochrome *c* give P/O = 2 or 1, respectively. These and

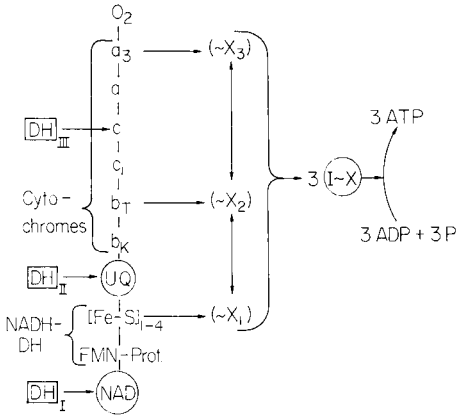

FIG. 4. Scheme of the energy transfer from the respiratory chain to ATP by energy-rich intermediates.

other results indicate that the energy-transfer sites or coupling sites at the respiratory chain are localized at the NADH dehydrogenase, at cytochrome *b* and at cytochrome a_3 (see Fig. 4). The first coupling site has a much lower molar occurrence ($\approx$1:10) than the second and third sites at the cytochrome level (cf. Fig. 1). Only the cytochromes occur in 1:1 molar ratio with the ATP synthase complex. We may speculate that the first coupling site associated with the dehydrogenase has a more special function.

The coupling sites at the respiratory chain have been also defined by the 'cross-over' points of oxido-reductive changes linked to the respiratory control (Chance & Williams 1956). More recently, the coupling sites have been studied for the apparent redox potential of the various components by 'clamping' the redox potential with redox mediators (cf. Wilson *et al.* 1973). The carriers that changed their redox state in these conditions under the influence of energy (ATP addition) are (Fe–S)-protein 'centres N-1 and N-2' of the NADH dehydrogenase complex (cf. Ohnishi 1973), cytochrome b_T and cytochrome a_3.

Reversal of oxidative phosphorylation and electron transfer

Electron transport can be coupled to the rate of oxidative phosphorylation. In terms of oxygen uptake this can result in the 'respiratory control' by the phosphorylation (cf. Chance & Williams 1956). In tight coupling conditions electron transport and energy transfer are reversibly coupled so that electron transport can be even reversed against the apparent redox potentials by use

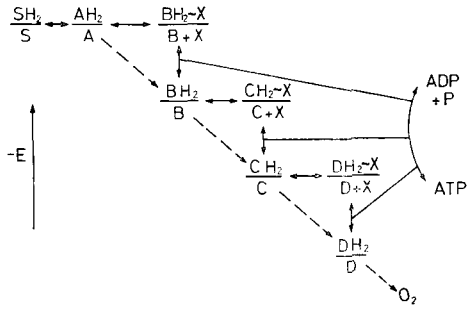

FIG. 5. The energy-dependent redox equilibrium extending over the respiratory chain as a result of the reversibility of oxidative phosphorylation (from Klingenberg 1968).

of energy from the phosphorylation. This reversed electron transfer was of great importance for the elucidation of the energy-transfer mechanism.

Reversed electron transfer was first observed as a reduction of NAD by ubiquinone-linked substrates in coupled mitochondria (Chance & Hollunger 1960; Klingenberg *et al.* 1959; Klingenberg 1960). Convincing evidence was obtained with the demonstration of hydrogen transfer to NADH-oxidizing substrates (Klingenberg & von Häfen 1963; Ernster 1961; Slater & Tager 1963). Complete reversal of oxidative phosphorylation starting from ATP could be demonstrated both in aerobic (Klingenberg & Schollmeyer 1961*a*) and, particularly impressively, in anaerobic conditions (Klingenberg & Schollmeyer 1961*b*; Chance 1961). These results lead to the 'redox patterns' of the respiratory chain and the concept of an ATP-linked or energy-linked redox equilibrium which extends over the respiratory chain in equilibrium with the ATP system (Fig. 5; Klingenberg & Schollmeyer 1961*c*). The relation between the redox states of the electron carriers and the free energy of the ATP system was quantitatively established. Recently, this energy-linked equilibrium was demonstrated as a function between the apparent redox potentials of the respiratory carriers and the phosphorylation potential (Wilson *et al.* 1973).

On the basis of the reversed electron transfer it has been possible to demonstrate the existence of intermediate energy forms which are not directly associated with any of the known components. In particular, the antibiotic oligomycin enabled a definition of this intermediate since it blocks the transfer of the energy to the ATP formation (Slater & Ter Welle 1969). Oligomycin does not inhibit reversed electron transfer, thereby indicating the participation of the intermediate energy form (Ernster & Lee 1964).

Reversed electron transfer also showed that the intermediate energy form is common to all three coupling sites. NAD can be reduced by succinate

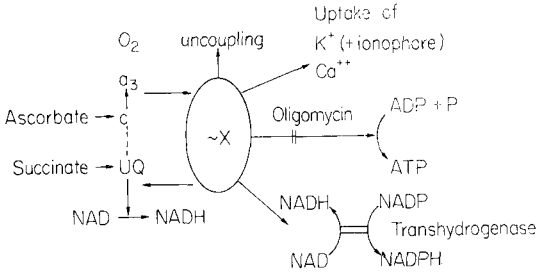

FIG. 6. The experimental definition of the intermediate energy form. Schematic summary of the experimental evidence demonstrating the existence of an energy form common to all coupling sites in the respiratory chain and its various pathways of use: reversibility of electron transport, transdehydrogenation, ATP synthesis, ion transport.

together with the oxidation of ascorbate and TMPD (Fig. 6) (Slater *et al.* 1962; Packer 1963). In the presence of oligomycin and absence of any ATP, the energy formed at the third coupling site can be used for reversed electron transfer at the first site. Another demonstration of energy transfer by intermediate energy forms comes from the energy-linked transdehydrogenation of NADH to NADP. This energy-dependent transdehydrogenation was first discovered for the endogenous NAD and NADP in mitochondria (Klingenberg & Slenczka 1959; Klingenberg *et al.* 1959) and then in sonic particles with exogenous nucleotides (Danielson & Ernster 1963*a*,*b*). The energy transfer from electron transport to the transdehydrogenation is insensitive to oligomycin.

Active ion transport by mitochondria

The most important demonstration of the existence of energy intermediates comes from energy-linked ion transport in the mitochondria (cf. Azzone & Massari 1973; Lehninger *et al.* 1967). Mitochondria can accumulate calcium at an impressive rate and degree. This accumulation depends only on the energy of respiration and, therefore, occurs also in the presence of oligomycin. More important is the active accumulation of K^+ in the presence of valinomycin or other ionophores. These ions are taken up at a high rate during active electron transport. The membrane contains an energy charge which can be released on the accumulation of these cations. The cation uptake is in most instances accompanied by release of H^+, which is of great significance for understanding the possible mechanism of the cation uptake. The H^+ release is largely abolished when permeant anions, such as acetate, are present; these anions accompany the movement of cations.

The stoichiometric relation between the cation uptake to respiration and to

H^+ release was determined and varies considerably with the conditions. The highest ratios K^+/O or Ca^{2+}/O should be most significant in reflecting the energy use for transport. Also, the ratio cation/H^+ ejection is of interest. A controversy about these values exists and ratios of $K^+/H^+ = 2$ or 1 have been given (Azzone & Massari 1973).

THE COUPLING MECHANISMS

So far, I have discussed the experimental outlines of the system of oxidative phosphorylation. For the mechanism of the energy transduction, I shall describe some further experimental data which relate more directly to the various proposed mechanisms. It is common to divide the theory of oxidative phosphorylation into three different concepts: (*a*) the chemical, (*b*) the conformational and (*c*) the chemiosmotic (electrochemical) coupling hypothesis. Other theories are current but I shall not discuss them here.

The chemical coupling

In the chemical coupling theory, the energy transduction was originally considered in analogy to substrate level phosphorylation (cf. Slater 1966). On oxidation of the substrate, an energy-rich covalent bond is formed with a hypothetical ligand $A_{ox}\sim X$. This chemical intermediate becomes independent of the electron carrier by combining with another ligand to form an intermediate $I\sim X$ which can react with P_i to form a phosphorylated intermediate $I\sim P$. This then reacts with ADP to form ATP (see equations 1–4).

$$A_{red} + B_{ox} + X \rightarrow A_{ox}\sim X + B_{red} \quad (1)$$

$$A_{ox}\sim X + I \rightarrow I\sim X + A_{ox} \quad (2)$$

$$I\sim X + P_i \rightarrow I\sim P + X \quad (3)$$

$$I\sim P + ADP \rightarrow I + ATP \quad (4)$$

$I\sim X$ corresponds to the energy-rich intermediate pool described above. Although the intermediate steps have been omitted, phosphorylated intermediates of electron carriers have also been postulated, for example, for ubiquinone. Although their existence has now been excluded, it is obviously more difficult to produce evidence for the existence of the $A_{ox}\sim X$ type intermediates with an unknown ligand. No chemical evidence for the existence of forms other than reduced and oxidized forms of the respiratory carriers has been presented so far. Some indirect evidence for energy-rich forms ($A_{ox}\sim X$ or $A_{red}\sim X$) has been deduced from apparent changes of the redox potential of some respiratory carriers under the influence of energy. In each of the three

coupling sites one component appears to shift its redox potential by about 200 mV, as illustrated in Fig. 2. These changes, as discussed by DeVault (1971), do not necessarily reflect covalent bonds but may also be caused by non-covalent ligands. It is, however, unsatisfactory that the energy-linked change of the redox potential for cytochrome a_3 is the opposite of that for cytochrome b_T; cytochrome b_T–ox and cytochrome a_3–red would, therefore, have to form the energy-rich compound. As a result, these effects have also been interpreted to reflect reversed electron transfer (Wikström 1973; Bonner *et al.* 1974).

A chemical form of the energy-rich intermediate state (e.g. I∼X) has not been identified. In principle, the phosphorylated energy-rich intermediate (I∼P) should be easily identifiable. For example, a phosphorylated protein in the ATP synthase complex might be assumed to be formed before ATP synthesis. However, no such intermediate linked to oxidative phosphorylation has yet been found despite considerable efforts.

More indirect evidence for possible P_i intermediates comes from $P_i/^{18}O$ exchange studies. This exchange depends on the presence of energy-rich intermediates and is, therefore, also sensitive to oligomycin. However, the fact that ADP must also be present suggests that ATP is formed in a concerted reaction from ADP and P_i (Boyer 1968).

Conformational energy transfer

Energy transfer by conformational changes has been discussed for the energy capture by the electron carriers, for their interaction with the ATP synthase and for the ATP synthase mechanism (Boyer 1965; Boyer *et al.* 1973; Green 1974). Conformational changes in energy coupling may be confined to one or two of these steps according to the various theories.

We can envisage conformational changes of components, which are restrained by binding in the membranes, used for energy transduction to some other components. As illustrated in Fig. 7A, both the oxidized and reduced form of the electron carrier can, in principle, attain both conformational changes, one of which is favoured more in the oxidized and the other in the reduced state. If the original conformation also is maintained on oxidation, an energy-rich state is stabilized by the restraints of the membrane.

One proposed redelivery of the energy is through the direct conformational interaction to the ATP synthase (Fig. 7B) (Ernster *et al.* 1974). For this interaction, two provisions are required: direct interaction between the electron carrier and the ATP synthase and also conformational changes in the ATP synthase as a mechanism of energy transfer.

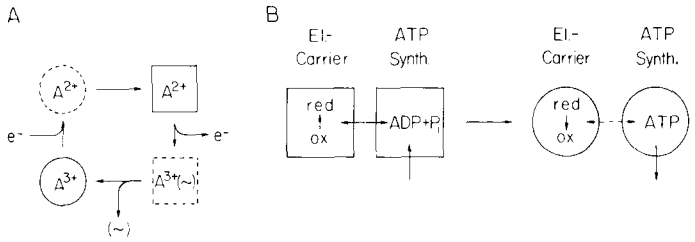

FIG. 7. Energy transfer from electron transport by conformational changes. A, Energy formation during an oxidation–reduction cycle of electron carrier by conformational change; B, energy transfer by conformational interaction through direct contact between electron carrier and ATP synthase.

The conformational theory as a basis for forming ATP in the ATP synthase complex has increasingly received attention. It was stimulated recently by a new insight into the energy transduction of actomyosin (Taylor 1973). The release of the binding may have a stronger conformational or energy transducing influence on the components than the chemical interconversion such as the ATP hydrolysis. Some evidence on the mitochondrial ATP synthase suggests that the release of the product ATP requires a major conformational change and energy-transducing steps (Boyer *et al.* 1973) (Fig. 8). The ATP synthase has been shown to contain tightly bound ADP or ATP which obviously can be dissociated only on coupling to energy transfer (Slater *et al.* 1974).

A model for energy transduction somewhat related to the conformational changes has been derived from the energy-linked changes in the mitochondrial structure (cf. Green 1974). However, these changes are relatively slow compared to the changes of energization measured by other criteria and, therefore, appear to be secondary effects. Furthermore, the changes do not allow us to draw any conclusion about the molecular conformational changes of the protein components.

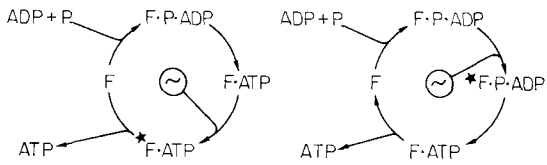

FIG. 8. Energy transfer for ATP formation in the ATP synthase. Left, energy transfer by activation of the ATP synthase–P_i–ADP complex for the subsequent formation of ATP; right, energy transfer required for dissociation of ATP from the ATP synthase. F = ATP synthase.

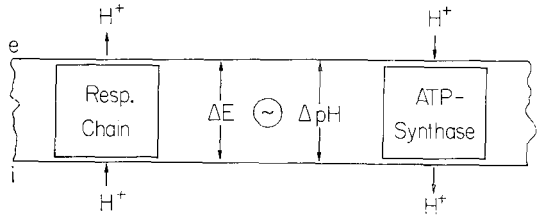

FIG. 9. Electrochemical (chemiosmotic) coupling between respiratory chain and ATP synthase. The energy-rich intermediate consists of an electrochemical H^+ gradient generated by an electrogenic transport of protons in the respiratory chain, used by return of H^+ via the ATP synthase. $\Delta\mu_{H^+} = RT\Delta pH - F\Delta\psi$, where $\Delta\psi$ is the potential difference across the membrane.

Electrochemical coupling

Electrochemical coupling as a mechanism for energy transfer from electron transport to ATP synthesis has received considerable attention in the last ten years (cf. Mitchell 1966; Greville 1969; Skulachev 1971). It has been called 'chemiosmotic' coupling although this early expression still rests on some concepts (i.e. an H^+ gradient instead of a membrane potential) which now have been largely overcome. This coupling mechanism requires that both the electron carriers and the ATP synthase are localized in a membrane, a fact which is not necessarily explained by the chemical or conformation energy transduction mechanism (Fig. 9). An important basis for these concepts rests in the former finding of energy-linked redox equilibrium in the respiratory chain (Klingenberg & Schollmeyer 1961*c*), for electrochemical coupling requires ready reversibility.

The energy-conserving mechanism is considered to consist of a build up of a H^+ gradient across the membrane. This electrochemical gradient should constitute the intermediate energy state (X or I~X) which, as in the chemical theories but in contrast to the consequent conformational hypotheses, is not directly associated with the electron carrier or the ATP synthase. The major aspect, and merit, of these concepts, therefore, has been the description of the energy-rich state. It requires the electron-transport chain to generate the electrochemical potential and that the ATP synthase uses its energy. This general picture appears to be quite widely accepted.

The proposed mechanistic and quantitative aspects of the coupling processes have been often modified in view of conflicting experimental data. The version most widely discussed is that the electrochemical potential across the mitochondrial membrane is generated by electrochemically active protons that are

pumped out from the matrix by the respiratory chain. In this process, a membrane potential positive outside is generated and a H^+ gradient is formed as a result of some countermovement of cations or parallel movement of anions (Mitchell 1966).

For ATP synthesis the energy is used in an electrogenic backflow of hydrogen ions. Experimental support comes from the reversibility of the ATP synthesis. Protons can be pumped out of the mitochondria on hydrolysis of ATP in the absence of electron transport (Mitchell & Moyle 1968). This reaction is sensitive to oligomycin; this is to be expected from Fig. 6. It illustrates that the ATP synthase can serve as an active pump for H^+. This reaction can be considered to be analogous to the ATP-driven cation pumps in other membranes.

The chemiosmotic hypothesis is limited at this stage in that it cannot provide a mechanism for the actual ATP synthesis which is rather a problem of the enzymology of ATP synthase. Some danger exists that chemiosmotic concepts distract attention from this basic enzymological problem.

Even modified versions of this hypothesis appear to disagree with the various quantitative aspects. In particular, a comparison of the free energy of ATP with the stoichiometry of H^+ movement to ATP synthesis and to electron transport is important. In Mitchell's version, a ratio $H^+/e^- = 1$ per coupling site is assumed, corresponding to $2H^+$ per one equivalent energy-rich intermediate. For the oxidation of NADH by the respiratory chain, $6H^+$/NADH should be pumped through the membrane which then should lead to the synthesis of 3ATP. The available experimental data, however, do not fully substantiate these postulates. Although some authors confirm the generation of $6H^+$/NADH oxidized (cf. Skulachev 1971), others report higher ratios (Papa *et al.* 1974; Azzone & Massari 1973). In particular, the data on K^+/H^+ or Ca/H^+, which closely correlate with the ratio H^+/e^-, appear to indicate a higher value (Azzone & Massari 1973). There is also one additional H^+ per ATP utilized in the exchange of ADP for ATP through the mitochondrial membrane which may consume in addition 0.6–1 H^+ per ATP extruded (see pp. 105–120).

The determination of the electrochemical potential across the inner mitochondrial membrane is experimentally very difficult. The measurements of the ΔpH across the membrane may be regarded with somewhat more confidence whereas the membrane potential measurements are controversial. Depending on the conditions, the ΔpH varies between 0.5 and 1.5 whereas the membrane potential ΔE (as measured by the gradient of K^+ or Rb^+ in the presence of valinomycin) lies between 100 and 200 mV (Rottenberg 1970). The largest part of the electrochemical potential consists of membrane potential difference.

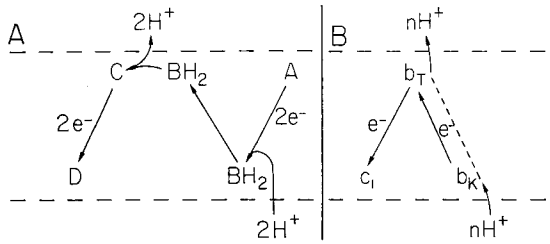

FIG. 10. Alternative mechanisms for the H^+ pump by electron carriers. A, Transport of H^+ by hydrogen carriers which, together with electron carriers, form a loop across the membrane; B, transfer of H^+ by vectorially arranged electron carriers via H^+-dissociating groups.

As the electrochemical potential energy should be equivalent to the free energy of ATP in equilibrium with the mitochondria, ΔG = 13–15 kcal (54–63 kJ), a considerably higher membrane potential (ΔE = 300 mV at ΔpH 1.5) would be required, with H^+/e^- = 1 per site. This dilemma could be solved if we accept that the ratio H^+/e^- is greater than 1 per coupling site as discussed above.

Another factor enabling ATP to be formed at electrochemical potentials smaller than the free energy of ATP comes from the 'active' extrusion of ATP through the mitochondrial membrane (see pp. 105–120). The endogenous ATP, which is in equilibrium with the electrochemical potential, has a free energy lower than the exogenous ATP by about 2–3 kcal (8–12 kJ).

Mechanism of generation of H^+ at the respiratory chain

The generation of H^+ according to the chemiosmotic hypothesis is based on a pair of hydrogen- and electron-transfer components at the coupling site (Fig. 10A), if we adopt an earlier model of Lundegardh (1945) and Robertson (1960). The hydrogen carrier picks up H^+ on the inside together with electrons and releases H^+ outside on oxidation with an electron acceptor. The hydrogen carrier, therefore, has to shuttle back and forth across the membrane. However, the electron pathway of the respiratory chain has to be rather distorted in order to accommodate these loops (Mitchell 1966).

Research on the respiratory chain does not support these schemes since there are no hydrogen carriers, for example, for the third coupling site. The only candidate which could reasonably be assumed to facilitate a hydrogen shuttle through the membrane is ubiquinone because of its lipophilic character. There is no evidence that even this component penetrates the membrane. However,

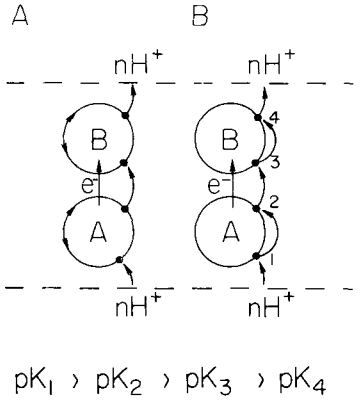

FIG. 11. Hydrogen ion channels through the membrane formed by electron carriers. H^+ transfer from carriers inside to carriers outside by H^+-dissociating groups (A) by rotational movements of the carrier or (B) by interaction of several groups in sequence on fixed carriers.

the asymmetric arrangement of the electron carriers across the mitochondrial membrane has been established and probably functions in the generation of the electrochemical potential.

The extrusion of H^+ through the membrane by other mechanisms has recently been considered. In particular, we can conceive of the change in the ionization constants of the respiratory carriers on the oxidation–reduction cycle being used as a force to drive the hydrogen ions (Wikström 1973). An influence of some ionizable groups in the cytochromes by oxidation–reduction reactions has been observed early, for example for cytochrome *b* (Urban & Klingenberg 1969). In this cycle there is no strict adherence to the $H^+/e^- = 1$ requirement as this ratio depends on the number of proton-dissociating groups influenced by the oxidation–reduction. Thus, this principle allows a ratio of H^+/e^- higher than the hydrogen-carrier loops.

In such a 'system', a H^+ channel through the membrane has to be provided. The transmembrane arrangement of the respiratory carriers can be considered to serve this purpose. As depicted in Fig. 11, a H^+ channel could consist of several H^+-dissociating groups of electron carriers in series from which H^+ is transferred, driven by the oxidation–reduction cycle (Klingenberg, unpublished results). This electron-driven H^+ channel is a prerequisite for any electrogenic movement of H^+. Recent schemes providing proton movement across the membrane with electron transfer from one point to the other in a direction perpendicular to the H^+ movement fail to satisfy these requirements. Here, the respiratory chain as a black box fails to explain an electrogenic H^+ movement (Papa *et al.* 1974).

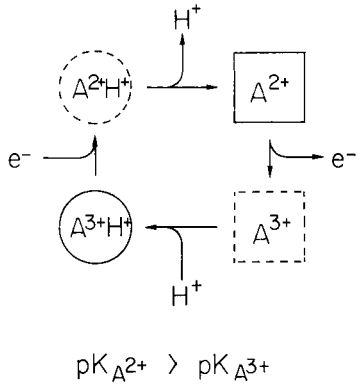

FIG. 12. Hydrogen ion dissociation of an electron carrier with conformational changes caused by the oxidation–reduction cycle.

A similar H^+ channel can be considered to exist also for the ATP synthase (Fig. 12). Here, no other possibility than the involvement of a H^+-dissociating group of the protein is conceivable. The driving force in this case is the ATP synthesis. In a different mechanism, Mitchell (1974) considers the electrogenic movements of H^+, associated with the channelling of ADP and ATP out of the active site, to be the driving force for the ATP synthesis. As the energy is conferred to the ATP formation, this mechanism does not consider conformational changes in the synthesis of ATP.

CONCLUSION

The mechanism of oxidative phosphorylation in mitochondria can be solved only by the isolation of the components and by investigation of their functional arrangements in the membrane. The most widely discussed theory of oxidative phosphorylation, the chemiosmotic hypothesis, fails to agree quantitatively with the data, although electrochemical coupling can be considered to be a valid overall mechanism of the coupling between the electron transport and the synthesis of ATP. The mechanism by which the electrochemical potential is generated in the electron chain is still unknown, although vectorial H^+ dissociation—so far ignored by the chemiosmotic theory—with conformational changes appear more satisfactory.

The synthesis of ATP can be considered primarily a problem of membrane-bound protein function, with its intrinsic implications such as lipid–protein interaction. Moreover, we should not neglect that some cytochromes and

ATP synthase are both composed of a surprisingly large number of different subunits, the function of which is not understood. Research on mitochondrial protein synthesis and the use of cytoplasmic or nuclear mutants of unicellular organisms (yeast, *Neurospora*) also provides apparently promising approaches to an understanding of the function of these complex proteins (Tzagaloff *et al.* 1973).

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For discussion, see pp. 63–68

Chloroplasts

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Abstract Illuminated chloroplast fragments, which can convert light into chemical energy ($NADPH_2$ and ATP), contain a number of soluble and insoluble electron carriers that have been arranged, on the basis of their redox potentials and on kinetic and other evidence, in sequences (analogous to those in mitochondria) to describe the events involved in the light reactions of photosynthesis. Fractionation of chloroplasts allows separation of two light-dependent partial reactions: the evolution of oxygen and the reduction of pyridine nucleotide, accompanied by ATP synthesis. The stoichiometry of the latter reaction is still uncertain.

Chloroplasts contain a directional proton-translocating ATPase (CF_1) needed for the phosphorylation of ADP in the light. The CF_1 also catalyses synthesis of ATP from ADP. When a pH gradient is applied in the dark across the phosphorylating membranes, the amount of ATP synthesized is related to both the change in pH and the electrical potential. During its catalytic activity, the CF_1 protein undergoes reversible conformational changes, but this is not the source of the driving force for ATP synthesis.

My purpose in this brief article is to outline current ideas about the mechanisms of conversion of light into chemical energy in chloroplasts (photosynthesis) and to give some examples of the experimental approaches which have led to our present picture of chloroplast function.

Chloroplasts (Fig. 1), occurring in the green parts of plants, are organelles enclosed by a double boundary membrane that encloses a lamellar (thylakoid) system supported in a stroma matrix. The thylakoid system may appear in the electron microscope to be differentiated into larger stroma lamellae and smaller stacked grana lamellae. Micrographs from the freeze-etch technique (Branton & Park 1967) show, from the pattern of the distribution of associated particles, that the membranes have a definite 'sidedness'. Light capture and

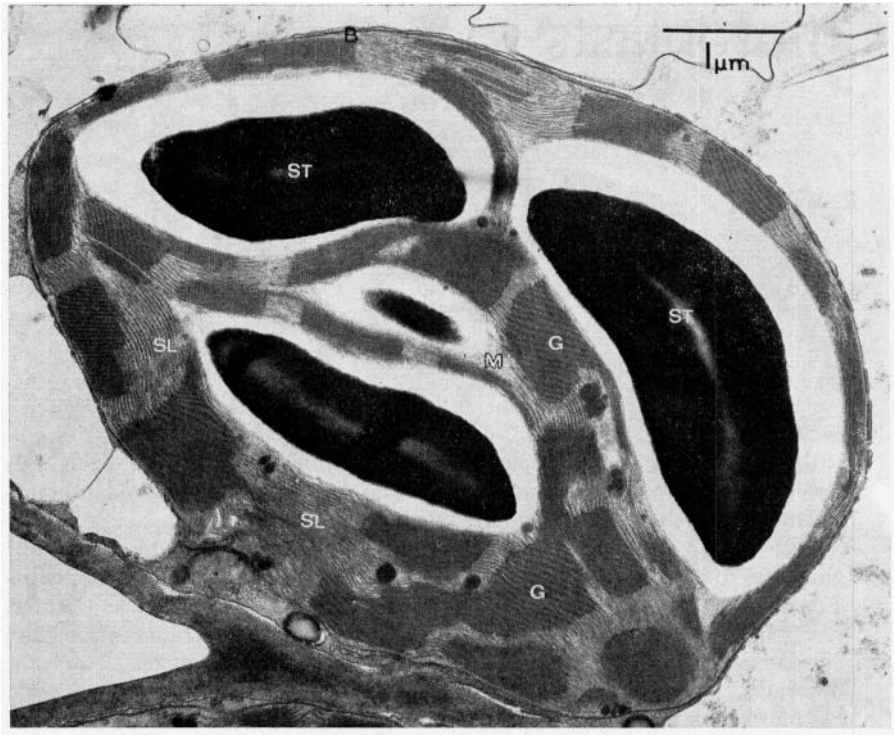

FIG. 1. Electron micrograph of chloroplasts of *Fittonia verschaffeldti*, fixed in glutaraldehyde and stained with OsO_4 and lead citrate (by courtesy of Dr J. M. Whatley): ST, starch grain; G, grana stack; SL, stroma lamella; B, boundary membrane; M, stroma matrix.

transduction occur in the thylakoid system; carbon dioxide is fixed and starch synthesized in the stroma matrix.

The part played by the chloroplast in the overall metabolism of the cell, and of the organism, is indicated in Fig. 2. On the left is represented the conversion of light into chemical energy, accumulated as reduced coenzyme ($NADPH_2$) and adenosine triphosphate (ATP), with water as the source of reducing electrons. Oxygen is discarded as a waste product. Secondary to this initial conversion of light into chemical energy is the storage of the accumulated energy in the carbon–carbon bonds of carbohydrates (or intermediates in their synthesis), a process which requires a supply of CO_2 and uses the $NADPH_2$ and ATP previously formed in the photochemical reaction. The apparatus for these conversions lies in the chloroplasts, where photosynthesis takes place. On the right is represented the release of chemical energy from the carbohydrates and its appearance in the energy-carrier ATP, a process which needs

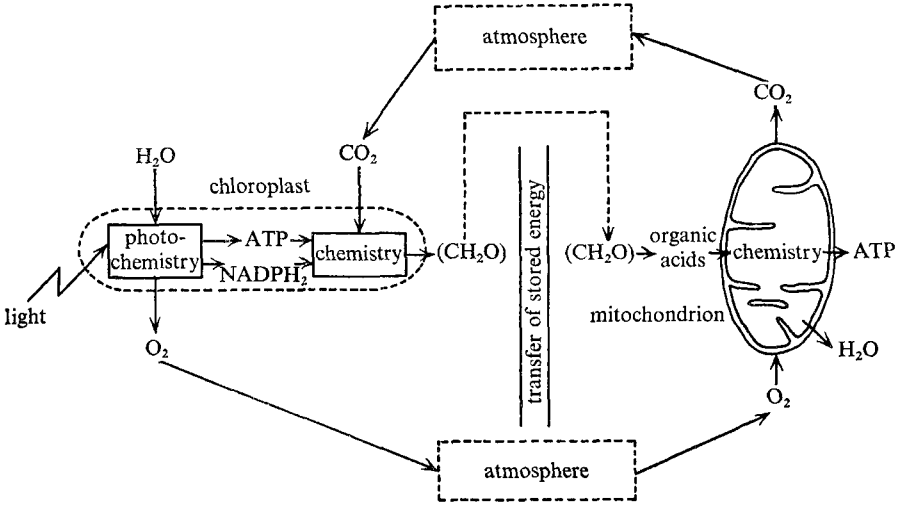

FIG. 2. The chloroplast in cellular metabolism.

a continuous supply of oxygen and which releases carbon dioxide as a waste product. The apparatus for these conversions lies in the mitochondria of plants and animals, where respiration takes place. The oxygen and carbon dioxide involved as substrates or waste products in respiration and photosynthesis are recycled through the atmosphere. The barrier between chloroplast and mitochondrion shown in the diagram indicates the need for the physical transfer of compounds containing stored energy from one organelle to another within a single cell, from one cell to another by way of translocation, or from one organism to another by way of a food chain. From the point of view of an individual cell, we are describing a miniature ecosystem, though the diagram itself applies equally well to a large scale ecosystem. The net effect of the integrated operation of this system is to make light energy supplied as an initial energy source available as usable chemical energy in the form of ATP for many metabolic syntheses both inside and outside the chloroplast. In Fig. 2 the biochemistry of the chloroplast is indicated as occurring in two 'boxes' labelled photochemistry (photochemical production of ATP and reduced pyridine nucleotide, $NADPH_2$) and chemistry (the bulk storage of the newly collected chemical energy). The extent of storage of light energy as chemical energy reaches a maximum at the time when only catalytic quantities of ATP and $NADPH_2$ have been formed; the subsequent use of these components in carbon dioxide fixation leads to a net loss of stored energy, although

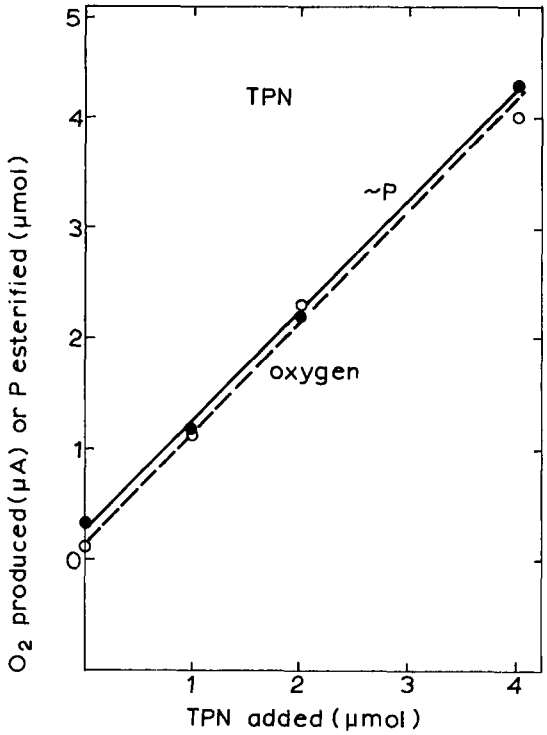

FIG. 3. Stoichiometry of oxygen evolution and ATP formation resulting from the photochemical reduction of TPN (NADP) (from Arnon *et al.* 1959).

the light energy converted can now be accumulated in bulk as suitable organic substances.

The overall photochemical reaction of photosynthesis, which is the essential energy transducing reaction of photosynthesis and the distinctive activity of the chloroplast, is shown experimentally in Fig. 3 (Arnon *et al.* 1959). The figure indicates that the reduction of pyridine nucleotide operates in specified conditions according to the stoichiometry in equation (1),

$$\mathrm{NADP} + \mathrm{H_2O} + \mathrm{ADP} + \mathrm{P_i} \xrightarrow[\text{ferredoxin}\\ \text{chloroplast}]{\text{light}} \mathrm{NADPH_2} + \mathrm{ATP} + \tfrac{1}{2}\mathrm{O_2} \qquad (1)$$

which describes a reaction sequence that is now called non-cyclic photophosphorylation. The second feature of this equation is that the reduction of

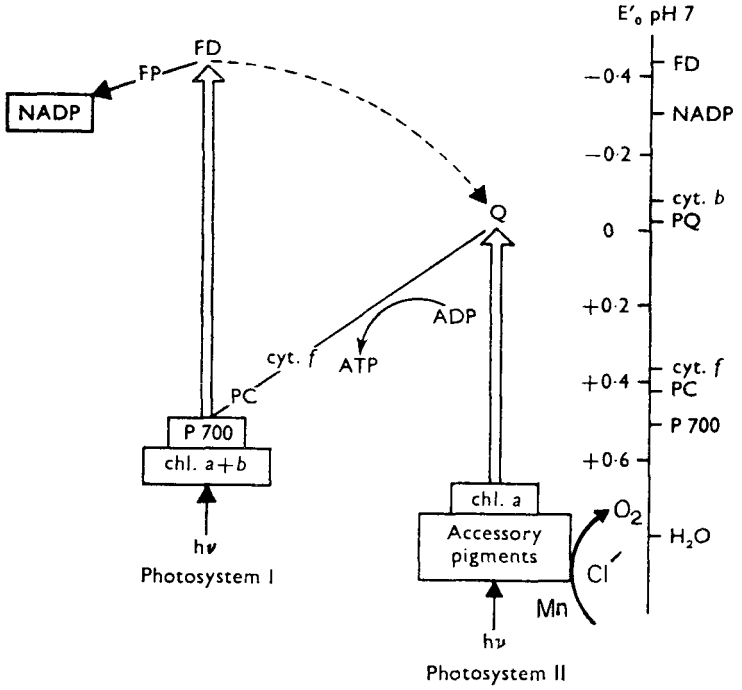

FIG. 4. A scheme for photosynthetic phosphorylation, showing the redox potential (E'_0/V) at pH 7 for several cytochromes, water and other carriers: FD, ferredoxin; PQ, plastoquinone.

pyridine nucleotide proceeds virtually to completion. This is initially unexpected since the reduction of NADP (redox potential, $E'_0 = -0.34$ V) by water ($E'_0 = +0.81$ V) is thermodynamically unfavourable in the dark. Moreover, the amount of energy needed for NADP reduction is not less than 230 kJ/mol (55 kcal/mol) (calculated at equilibrium for both half reactions) and we must further allow for the accumulation of at least one mole of ATP (about 50 kJ/mol [12 kcal/mol] for its formation). The amount of energy (190 kJ/einstein, 45 kcal/einstein) available in red light at 680 nm, a wavelength that is still photosynthetically efficient, is clearly too small for one quantum to bring about the overall reaction. Equation (1), therefore, implies the cooperation of two or more quanta to raise electrons from the redox potential of water to that of pyridine nucleotide and it has been shown that for each NADP reduced four quanta must cooperate (two quanta per electron).

Fig. 4 shows a simple scheme, based on the redox potentials of the carriers that have been identified, to describe the possible process of energy transduction.

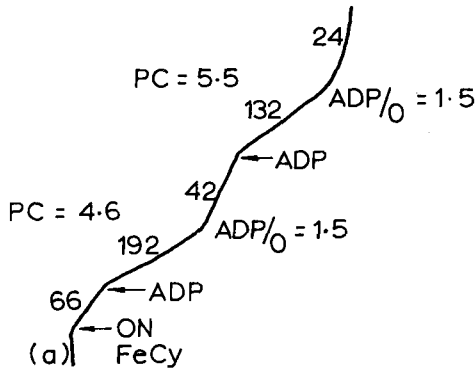

FIG. 5. Photosynthetic control with ferricyanide (FeCy; added at the same time as turning on the light). The figures along the traces are μatom oxygen/(mg chlorophyll h): PC, photosynthetic control ratio; ADP/O, μmol ADP added/μatom oxygen evolved. (From Reeves *et al.* 1971).

Light is captured by the pigment chlorophyll *a* and the accessory pigment, usually chlorophyll *b* in green plants. Two photochemical reactions are thought to be involved (photosystems I and II) and these are shown as being joined by a thermochemical bridge allowing them to operate in sequence. Transfer of electrons along the thermochemical bridge is accompanied by the formation of ATP. The electron donor is water and the most reduced electron acceptor is ferredoxin, which in a dark reaction catalysed by a flavoprotein reductase (FP) yields the $NADPH_2$. A number of alternative artificial electron acceptors can be used experimentally to substitute for ferredoxin, including various reducible dyes, quinones and ferricyanide; it is also possible to set up an experimental system in which the terminal electron acceptor is H^+. In the presence of the enzyme hydrogenase, protons can accept electrons to yield molecular hydrogen and, in the presence of chloroplasts and added ferredoxin, a photochemical evolution of H_2 that is coupled to ATP synthesis is possible (Arnon *et al.* 1961*b*).

A shortened electron flow between ferredoxin and cytochrome *f* driven only by photosystem I yields ATP but no net oxidation–reduction product. This is termed cyclic photophosphorylation.

The rate of electron transport to pyridine nucleotide is affected by the presence or absence of the phosphorylating system and there is an enhancement of the rate of reduction of pyridine nucleotide in the presence of ADP and inorganic phosphate, provided the system is not limited by any externally added component. This observation led to a demonstration of the occurrence of photosynthetic control (analogous to respiratory control in mitochondria) in which the rate of reduction of a terminal acceptor (ferricyanide in Fig. 5) is

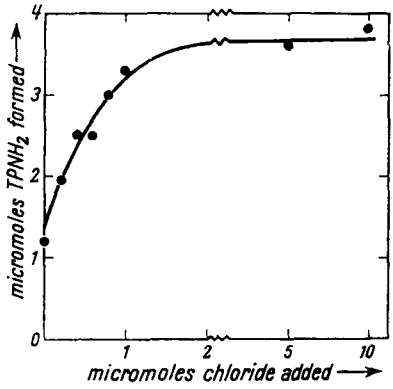

FIG. 6. Effect of chloride on photoreduction of NADP (TPN). (From Bové *et al.* 1963).

stimulated by the addition of ADP in the presence of phosphate and which reverts to the unstimulated rate when all the ADP has been phosphorylated to form ATP (West & Wiskich 1968; Reeves *et al.* 1971). In Fig. 5, the ratio between the stimulated and unstimulated rate (the photosynthetic control ratio) is 4.6–5.5. The observed ADP/O ratio is about 1.5. This raises the question whether the stoichiometry of the reaction is correctly described by equation (1). A number of workers (see summary by Trebst 1974) believe that more than one ATP can be formed during the reduction of one mole of pyridine nucleotide or its equivalent (e.g. 2 mol ferricyanide). A difficulty in accepting this view is the possible cyclic photophosphorylation at the same time as a non-cyclic electron transport. Although, clearly, conditions can be arranged in which the stoichiometry of NADP to ATP of 1:1 can be regularly obtained, equally, conditions can be arranged in which the measured ratio is greater than 1. The final conclusion of this question must await further experimentation.

Numerous experimental approaches have been used to support the proposals shown in Fig. 4, including conventional biochemical experiments with removal and replacement of suspected intermediates, spectroscopic experiments, kinetic experiments with continuous white or monochromatic light and the cooperation of different wavelengths of light, experiments with electron spin resonance and other physical measurements, and experiments with antibodies which show the sidedness of the energy-transducing membranes.

An example of conventional biochemistry involving the simple removal and replacement of a soluble component has demonstrated that chloride ions are necessary for the evolution of oxygen. Chloride is removed from most chloroplasts simply by washing and can be readily added back, as is shown in Fig. 6

(Bové *et al.* 1963). If oxygen cannot be evolved (i.e. when water cannot act as donor), pyridine nucleotide cannot be reduced. It is then convenient to measure the effect of chloride indirectly by measuring the reduction of pyridine nucleotide. Surprisingly, a high concentration of chloride is needed for the photoreduction of pyridine nucleotide. It was also demonstrated that in the absence of chloride, when the production of pyridine nucleotide is not possible, carbon dioxide cannot be reduced to intermediates of carbohydrate synthesis (Bové *et al.* 1963).

The scheme in Fig. 4 indicates a requirement for plastoquinone in the transport of electrons between the two photochemical reactions. This requirement has been biochemically demonstrated by experiments in which quinones were exhaustively extracted from lyophilized chloroplasts. Extraction leads to loss of ability of the chloroplast, when resuspended in a suitable mixture, to reduce pyridine nucleotide or to evolve oxygen (Fig. 7*a*). The addition of plastoquinone to the inactive extracted chloroplasts restores the ability to evolve oxygen and to reduce pyridine nucleotide, but plastoquinone cannot be replaced by ubiquinone, tocopherol quinone, vitamin K or its derivatives, or by a number of benzoquinones (Krogmann & Olivero 1962; Arnon & Horton 1963). The ability to extract most of the quinone from lyophilized chloroplasts without impairing the reduction of pyridine nucleotide while preventing the use of ferricyanide as an acceptor has led to some slight confusion on this point. Pyridine nucleotide can, however, be reduced when other electron donors replace water, such as reduced 2,6-dichloroindophenol maintained in the reduced state by ascorbate (Vernon & Zaugg 1960). Chloroplasts from which the plastoquinone had been extracted could no longer use water as an electron donor but with reduced indophenol dye as donor they were perfectly competent to photoreduce pyridine nucleotide (Fig. 7*a*). This supports the location of plastoquinone as an acceptor of electrons from photosystem II, although it is clearly some way distant from being a direct donor of electrons to photosystem I. If the site of action of plastoquinone was bypassed through the alternative electron donor (indophenol), the operation of photosystem I was easily demonstrated. This might represent a biochemical separation of the two partial reactions of photosynthesis, a suggestion that has been challenged subsequently. Fig. 4 suggests that cyclic photophosphorylation, which bypasses photosystem II and depends on photosystem I only and which leads only to the synthesis of ATP, also depends on plastoquinone as an intermediate catalyst. If this is so, the extraction of plastoquinone should remove the capability of chloroplasts for cyclic photophosphorylation; this was demonstrated (Fig. 7*b*) with three artificial carriers of cyclic phosphorylation, phenazozonium methosulphate, vitamin K_3 and flavin mononucleotide. Some interest-

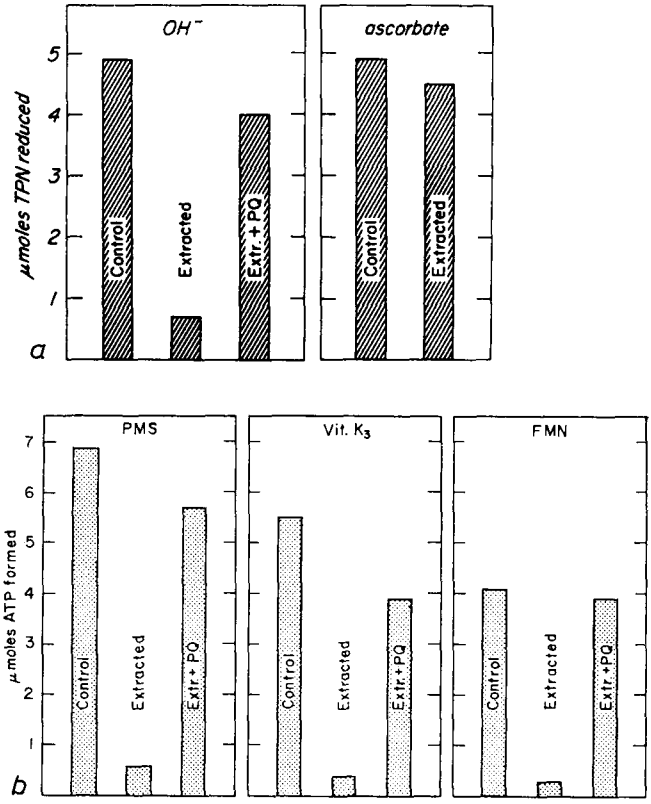

FIG. 7. The role of plastoquinone (PQ) in chloroplast reactions: *(a)* alternative electron donors, showing requirement for PQ only with water (OH^-) as donor for NADP (TPN) reduction; *(b)* requirement for PQ in several cyclic photophosphorylations: PMS, phenazonium methosulphate; FMN, flavin mononucleotide. (From Arnon & Horton 1963 and Whatley & Horton 1963).

ing data by Krogmann & Olivero (1962) indicate that electron transport can be restored by derivatives of plastoquinone with shortened isoprenoid side-chains but that the restoration of phosphorylation depended on the presence of a longer isoprenoid side-chain. This suggests the direct involvement of plastoquinone, shown merely as an electron transport catalyst in Fig. 4, in energy transduction leading to ATP synthesis. An alternative suggestion by Trebst (1974) is that the extraction of plastoquinone makes the membranes leaky to protons, thus preventing ATP synthesis, and that only the plastoquinones with longer side-chains can effectively restore the impermeability of the membrane.

Fig. 4 also suggests that cytochromes participate in the thermochemical part

of the electron-transport chain. Spectroscopic evidence, ranging over a considerable degree of complexity, has permitted the direct detection of cytochromes. The early observations of cytochromes in acetone powders of leaves and chloroplast preparations (Hill & Scarisbrick 1951) were made with a hand spectroscope, but later work used difference spectra and kinetic measurements (e.g. Bendall *et al.* 1971). Even more sophisticated work has involved the fitting of experimental results by curve generators and the analysis of the curves up to fourth order differentials (Shipp 1972). Cytochrome *f*, a cytochrome of the *c* type found only in chloroplasts, is closely associated with the operation of photosystem I. The photooxidation of the related photosynthetic cytochrome c_2 in the photosynthetic bacterium *Chromatium* has been observed both at room temperature and equally at liquid nitrogen temperature (77 K) in frozen preparations (Chance & Nishimura 1960); a similar demonstration for cytochrome *f* has been made for green plants (Witt *et al.* 1961; De Vault & Chance 1966). Since conventional collision chemistry is ruled out at 77 K, the basis of the photochemical reactions is clearly a physical transfer of the electron from the oxidized cytochrome to a photoactivated chlorophyll molecule, which then is able to release the electron to a suitable acceptor molecule. Because the mechanism of photooxidation of the cytochrome is essentially a physical one it requires the close structural apposition of the active centre of the chlorophyll and of the cytochrome *f* molecule.

Cytochromes can be measured from their difference spectra by titration with various redox buffers (Bendall *et al.* 1971); in this way cytochromes *f*, b_6 and b_{559} have been detected in chloroplasts from plants such as the French bean, *Phaseolus vulgaris*. Difference spectra have been obtained both at 77 K and at room temperature. Both the total amount and relative amounts of the various cytochromes change during the greening of chloroplasts from an etioplast (see Boardman *et al.* 1971; Whatley *et al.* 1971; Plesnicar & Bendall 1971). Fig. 8*a* shows the spectral development of cytochrome components during the greening of bean chloroplasts and Fig. 8*b* shows the results of an experiment in which the various components were identified on the basis of redox titrations. During the greening of chloroplasts the ability to do cyclic photophosphorylation (photosystem I) appears earlier than the ability to reduce pyridine nucleotide at the expense of water as electron donor (photosystem I and II). However, photosystem II is probably effective early in greening, as judged by the use of the multiple flash technique (Gyldenholm *et al.* 1971). It has, therefore, been concluded that the critical requirement during development for a coupling between photosystems I and II is the formation of the high potential form of cytochrome b_{559} (Fig. 8*b*).

In kinetic experiments on cytochrome *f*, photooxidation and photoreduction

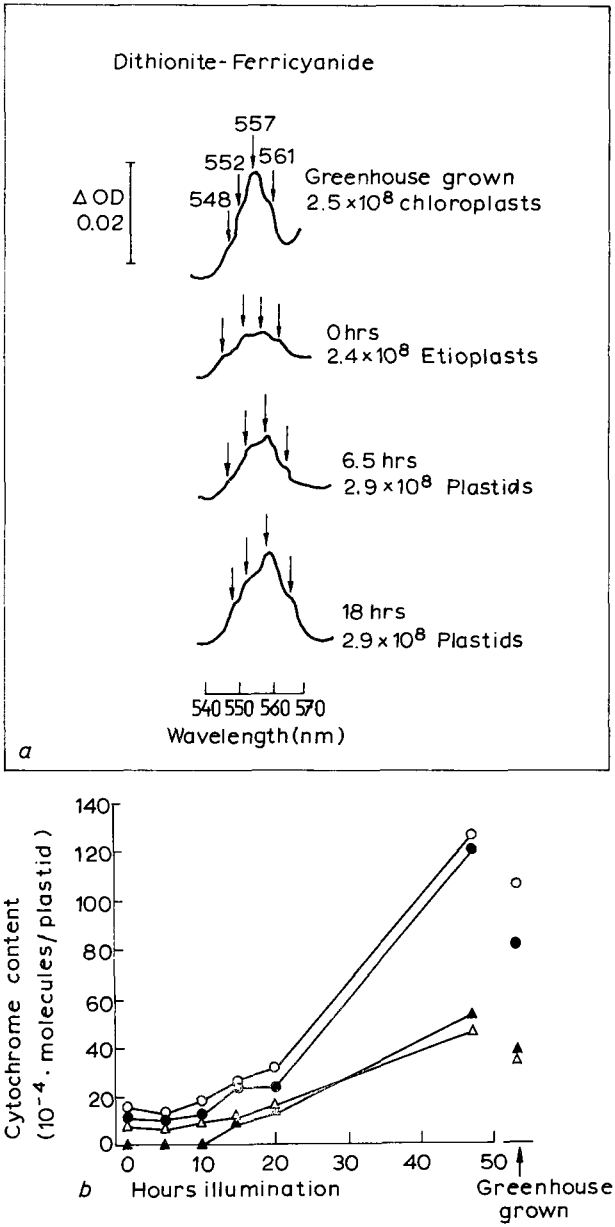

FIG. 8. Development of cytochromes during greening of bean chloroplasts. *(a)* Difference spectra at 77 K; the shoulder at 552 nm corresponds to cytochrome *f*, at 557 nm to cytochrome b_6 and at 561 nm to cytochrome b_{559} (note effect of low temperature measurement on absorption maximum). (From Whatley *et al.* 1971). *(b)* The cytochrome content of the plastids of the primary leaves of 14-day-old dark-grown beans during the first 48 h of greening under an illumination of 700 μW/cm² at 25 °C: ▲, cytochrome *b*-559 $_{HP}$; ○, cytochrome *b*-559$_{LP}$; ● cytochrome *b*-563; and △, cytochrome *f*. (From Gregory & Bradbeer 1973).

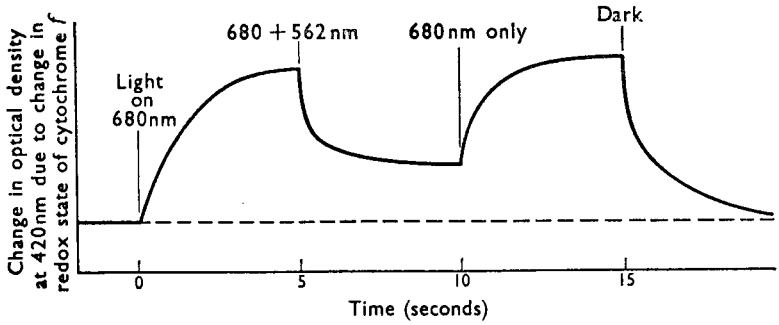

FIG. 9. Oxidation and reduction of cytochrome *f* in the red alga *Porphyridium*. Increase in optical density at 420 nm is due to oxidation and the decrease is due to reduction of cytochrome *f*. Light of 680 nm is absorbed by photosystem I (chlorophyll *a*) and at 562 nm is absorbed by photosystem II (phycoerythrin). (From Duysens & Amesz 1962).

have been measured with a red alga *Porphyra tenera* (Duysens & Amesz 1962). This organism has phycocyanin and phycoerythrin instead of chlorophyll *b* as its accessory pigments. Therefore, with monochromatic light one can illuminate either chlorophyll *a* (680 nm), so that photoreaction I is principally activated, or the phycobilins (562 nm), so that photoreaction II is principally activated. As shown in Fig. 9, light of 680 nm causes the oxidation of cytochrome *f*, whereas the addition of light at 562 nm causes its partial reduction. When the 562 nm light is turned off, the oxidation of cytochrome *f* proceeds again and when the algae are returned to the dark the cytochrome *f* becomes fully reduced. This experiment implies the operation of two photoreactions, one of which (photosystem I) removes electrons from the intermediates of the thermochemical bridge, and the other (photosystem II) which adds them to the intermediates. The redox state of any component which operates between the two photochemical reactions then depends on the relative activities of the two photochemical reactions.

Fig. 10 shows the action spectrum for the green alga *Ulva taeniata* in which the rate of photosynthesis is compared with the amount of light absorbed as a function of the wavelength of the light supplied. There is a fairly good agreement between light absorbed and photosynthetic efficiency over most of the wavelength span except in the region 460–500 nm (where carotenoids absorb light that is not photosynthetically effective) and beyond 700 nm, where the two curves separate. The efficiency of the light absorbed falls markedly beyond 700 nm, a phenomenon now termed the red drop. The light absorbed beyond 700 nm is, however, still available for photosynthesis. Thus, if to light beyond 700 nm is added light of a shorter wavelength the photosynthetic product is

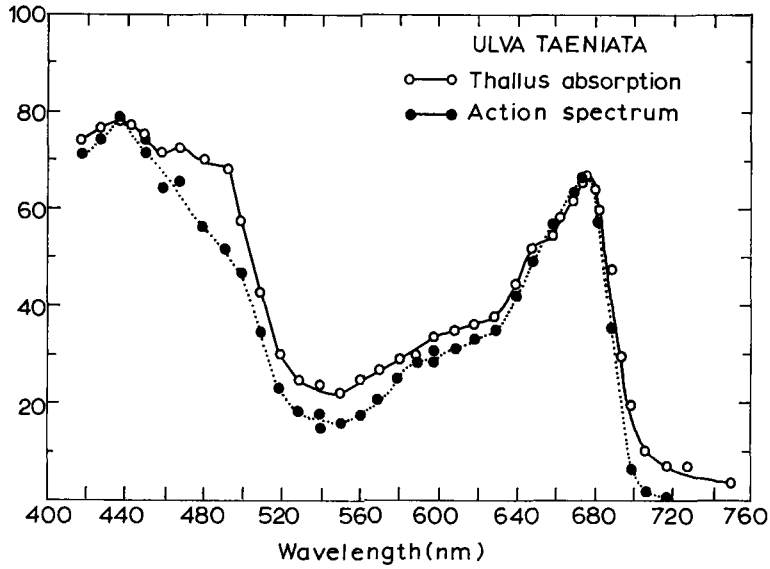

FIG. 10. Absorption (○—○) and photosynthetic action (●····●) spectrum of *Ulva taeniata*. (From Haxo & Blinks 1950).

greater than the arithmetic sum of the photosynthetic products of two wavelengths supplied separately. The enhancement obtained (Emerson *et al.* 1957) is now interpreted as a cooperative effect between the two photochemical systems. Also based on the Emerson enhancement effect are the so-called chromatic transients, where the effective intensity of the light is maintained but its spectral composition is changed. When Myers & French (1960) applied chromatic transients to algae in an oxygen electrode, they observed both temporary overshoot and temporary undershoot on the rate of oxygen evolution when the wavelength of the light was changed. This is consistent with a cooperation between two photochemical reactions. Even more remarkably, Myers & French found that the photosynthetic system had a 'memory' in that it was possible to delay the application of the second light in the series for various times: the system still 'remembered' the wavelength of the other light to which it had most recently been exposed. On the basis of Duysens' experiments, this can be interpreted to mean that electrons are either accumulated in or depleted from components of the photochemical bridge by particular light treatments and that the memory is the result of their accumulation in or loss from organic molecules. Loss of memory would then be due to loss of electrons to acceptors outside the photochemical reactions or their supply

from non-photochemical sources. The total time during which the memory could still be detected was many seconds.

The experiments depending on light quality so far noted have all been 'physiological' ones with whole organisms. A similar effect of different wavelengths of light has also been shown for isolated chloroplasts, in which the effect of altering the wavelengths clearly demonstrates a change in emphasis between cyclic and non-cyclic modes of electron transport leading to ATP synthesis. Since cyclic electron flow requires only the participation of photosystem I, and since photosystem I is driven by chlorophyll *a* (P_{700}), it is effective at much longer wavelengths than the non-cyclic system which requires the cooperation of photosystems I and II (Arnon *et al.* 1961*a*).

Some interesting experiments by Joliot *et al.* (1969) and Kok *et al.* (1970) have also indicated an electron-storage system associated with photosystem II. The yield of oxygen resulting from the application to a chloroplast suspension of a series of brief but intense flashes of light was measured: the yield of oxygen per flash depended on the number of flashes that had preceded it. Thus, a single flash applied after a period of darkness gave no yield. Flash 2 gave some oxygen and flash 3 gave a maximal yield, whereas flash 4 yielded a smaller amount of oxygen. If the flashing was continued oxygen was found to peak at flashes 3, 7, 11, after which the effect became less apparent. This is interpreted to indicate the necessary cooperation of four quanta per oxygen molecule evolved, which agrees with the earlier observation on the stoichiometry of non-cyclic photophosphorylation.

Further evidence for two photoreactions was obtained from electron spin resonance measurements (Weaver 1968). On illumination, the unpaired electrons generated in organic molecules by photochemical reactions could be detected at the characteristic signal at $g = 2.003$. If the peak of the e.s.r. signal was monitored, the appearance of this signal on illumination could be shown; on turning off the light the decay of the signal could be measured. There was a clear difference between two components during the decay, a 'fast' component (which decayed quickly) associated with the acceptor of photosystem I and a 'slow' component (which decayed slowly) associated with plastoquinone, the acceptor of photosystem II. E.s.r. measurements have also contributed to investigations of the role of manganese in oxygen evolution. Although physiological experiments (e.g. those of Kessler on *Ankistrodesmus*) indicated a close correlation between lack of manganese and an inability to use water as an electron donor, isolated chloroplasts which are known to contain manganese show no e.s.r. signal. This indicates the absence of Mn^{2+} ions free to interact with the water molecules in the solution. When the pH of the medium is lowered, the characteristic e.s.r. signal of Mn^{2+} appears at the same time as the

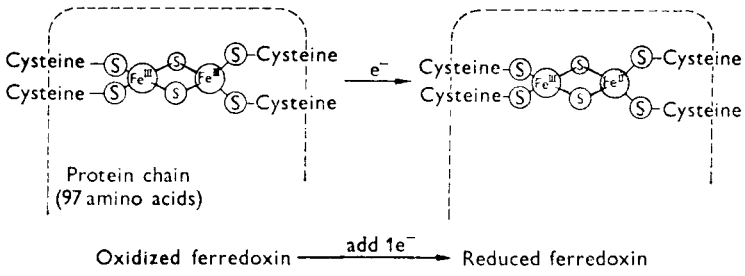

FIG. 11. A model for the active centre of plant ferredoxins, showing the oxidized and reduced forms of the protein. (From Rao *et al.* 1971).

ability to evolve oxygen disappears. This suggests that the manganese is occupied in a chelate complex; since Mn^{2+} can undergo redox changes it is an attractive possible component of the oxygen-evolving system but there is no direct evidence that it is thus involved (Cheniae 1970). However, it has been proposed that chloride participates as a stabilizing ion during the redox function of metal complexes and this may be the role of chloride in oxygen evolution where it may function in association with manganese.

The terminal acceptor of electrons according to Fig. 4 is soluble ferredoxin. Though it is known that soluble ferredoxin is the first stable reduced product of photosynthesis and that its subsequent reoxidation leads to NADP reduction, it has not been verified that it is the most reducing electron acceptor; the ferredoxin reducing substances proposed by Trebst and San Pietro are possibly interposed. Moreover, other insoluble non-haem iron proteins (ferredoxins) have been identified in chloroplasts and photosynthetic bacteria, which have redox potentials considerably more reducing than the soluble ferredoxin of Fig. 4, and which may themselves prove to be intermediate acceptors of electrons from photosystem I prior to soluble ferredoxin (see Trebst 1974, especially p. 427).

Spinach ferredoxin is a small protein (molecular weight 12 000) which contains two iron and two labile sulphur atoms per molecule (released as H_2S on acidification) but no haem groups. Its redox potential (E'_0 -0.42 V) is the same as that of hydrogen at pH 7 (Arnon 1969; Hall & Evans 1969). The physical properties of spinach and other ferredoxins have been investigated in some detail with e.s.r., optical rotatory dispersion and Mössbauer spectroscopy. The constituent amino acids have been identified and sequenced. On the basis of this extensive evidence the structure of the chromophoric group, which is also the active centre, has been deduced to be as shown in Fig. 11. Spinach ferredoxin is known to accept a single electron on becoming reduced and to

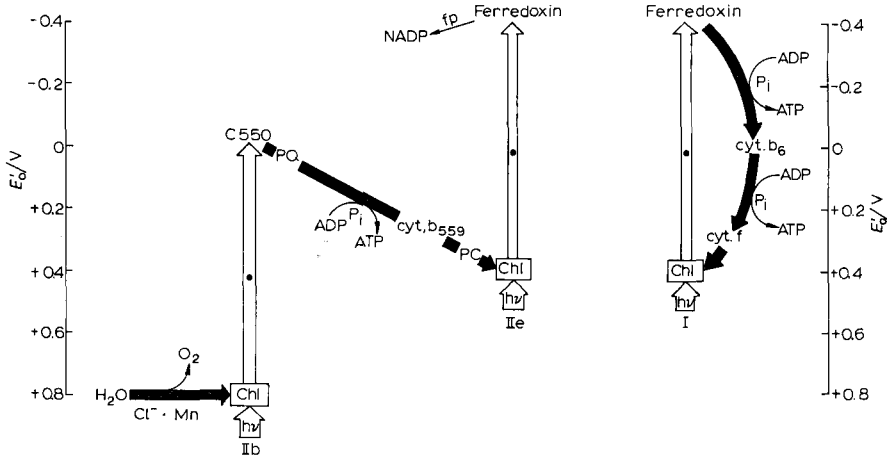

FIG. 12. A model suggesting the complete separation of cyclic and non-cyclic photosphosphorylation in chloroplasts (Knaff & Arnon 1969).

donate this electron to the flavoprotein ferredoxin NADP-reductase, which accumulates pairs of electrons for onward transmission to NADP. Ferredoxin forms a 1:1 complex with the reductase, by which it can be attached to the thylakoid membrane. Consistent with the view that ferredoxin can act as the terminal electron acceptor is the observation that added ferredoxin is reduced on illumination in a mixture containing chloroplasts and that, on subsequently turning off the light, it is reoxidized at the expense of oxygen generated during its initial photoreduction by water (Arnon *et al.* 1964).

Another approach to the separation of the activities of photosystems I and II has been the attempt to separate chloroplasts into fractions differentially enriched with respect to the two photosystems. By the use of detergents, sonication and other types of biochemical procedures suitable for the analysis of membrane structures (Boardman 1970), the spinach chloroplasts have been separated into fractions catalysing partial reactions of photosynthesis. Subsequently, Arnon and his colleagues, applying essentially similar treatments, claim to have isolated two preparations, one of which can cyclic phosphorylate but is incapable of non-cyclic phosphorylation and the second, which can perform only non-cyclic phosphorylation. This work, taken together with other lines of evidence, led to the formulation in Fig. 12 (Knaff & Arnon 1969), which suggests the physical separation of the two complete phosphorylation systems, whereas Fig. 4 involves only two partial photochemical reactions, whose operation to give either cyclic or non-cyclic photophosphorylation

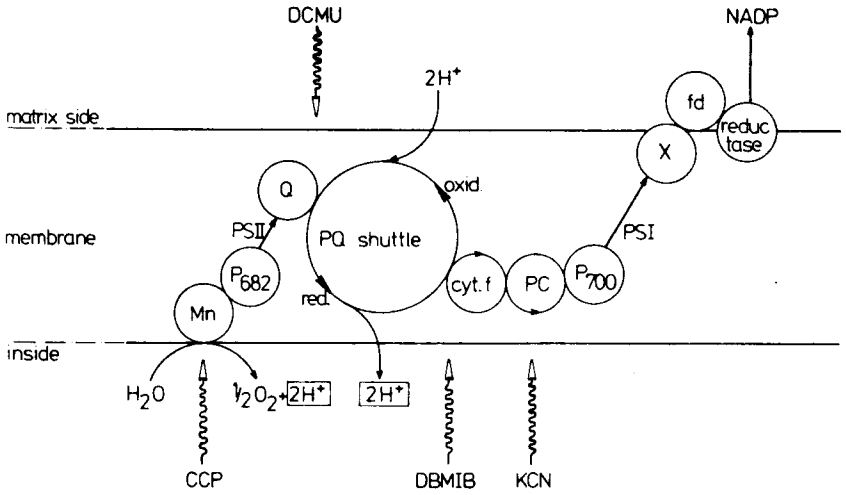

FIG. 13. Photosynthetic electron flow from water to NADP across the membrane. Two proton-releasing sites inside the membrane are indicated, as also are the relative positions of a number of components of the electron transport chain. The points of action of a number of important inhibitors are also indicated (from Trebst 1974): CCP, carbonyl cyanide phenylhydrazone; DBMIB, dibromothymoquinone; DCMU, dichlorophenyldimethylurea.

depends on experimental or physiological conditions. Park & Sane (1971) suggest that there may be a partial physical separation in nature between the lamellar structures capable of performing one or other of the processes and that, in spinach chloroplasts, cyclic phosphorylation may be restricted to the stroma lamellae, although this cannot be universally true since not all chloroplasts have a differentiation into stroma and grana lamellae.

A different aspect of energy transduction in chloroplasts is emphasized by a spatial model instead of a redox model. Experiments with antibodies against specific chloroplast components, taken together with much other evidence, indicate clearly the sidedness of photosynthetic membranes. Trebst (1974), therefore, proposes a spatial model (Fig. 13) which shows the reduction of pyridine nucleotide coupled to the pumping of protons from one side of the membrane to the other and indicates a strong sidedness of the membrane itself, with some components orientated at the inner and others at the outer surface. These proposals, as well as the redox formulations of Figs. 4 and 12, are supported by the kinetic analysis of Witt and his colleagues (see Witt 1971) who have spectroscopically monitored changes in many of the components of the membrane system and obtained information on the sequence of events after illumination. The earliest event appears to be a polarization of the mem-

brane, which is detected by absorption changes resulting from the effect of the electrical field on the orientation of carotenoids, followed relatively slowly by the formation of a pH gradient by proton pumping. Redox changes in many components, including cytochromes and plastoquinone, were also detected and their kinetics were measured.

The ability to pump protons is a property common to chloroplasts, mitochondria and bacteria. Broken chloroplast fragments (thylakoid membrane preparations), submitochondrial particles, phosphorylating particles from aerobic bacteria and bacterial chromatophores resemble each other in pumping protons from the medium into the vesicles and all synthesize ATP at a membrane-bound ATPase which is located at what is now the outer surface of the membrane (recognized as a stalked particle in electron micrographs after negative staining). This orientation of ATPase and the direction of pumping protons is reversed in intact mitochondria, aerobic bacteria and photosynthetic bacteria, so that these all exude protons into the medium and synthesize their ATP internally. The thylakoid membrane system, however, retains its 'inverted' configuration within the intact chloroplast, so that it continues to abstract protons from the medium (the stroma enclosed by the boundary membrane of the chloroplast) and synthesizes ATP at its external surface, where the ATP is available directly for synthetic purposes in CO_2 fixation and other processes in the chloroplast. That is, the chloroplast membrane system resembles in its configuration not the mitochondrion but the submitochondrial system. These relationships are summarized in Fig. 14.

The chemiosmotic hypothesis (Mitchell 1970) requires that ATP-synthesizing membranes should have distinct sidedness and be essentially impermeable to protons. Thus, by the setting up of proton gradients with electron-transport shuttles driven either photosynthetically or at the expense of substrate oxidation, an electromotive force is generated which can be coupled to the formation of ATP when protons are transported through the membrane-bound ATPase. In this connection the so-called 'acid bath' experiment of Jagendorf & Uribe (1966) is of special interest. Chloroplasts are suspended in the dark in a medium of low pH, so that the internal pH of the chloroplast becomes close to that of the medium after a period of equilibration. Subsequently, ADP and P_i are added, followed by sufficient alkali to raise the pH of the external medium to 8.5. This creates a proton-motive force owing to the change of pH and protons now tend to exit from the chloroplast into the medium to restore equilibrium. They may do so slowly by reversal of the slow permeation observed in the first part of the experiment, or rapidly through the ATPase system in the membrane, so that ATP is generated. In this way chloroplasts, which usually depend on light to generate the pH differential, can be made to synthesize ATP in

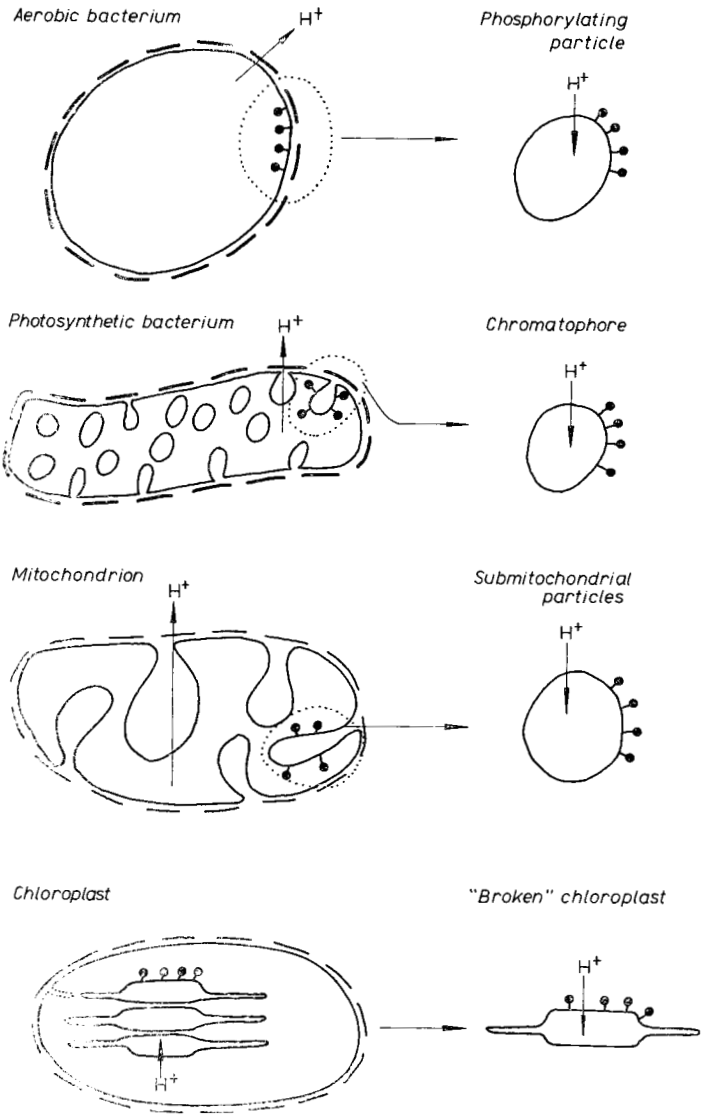

FIG. 14. Relation between membrane sidedness and the direction of pumping of protons (Fig. by courtesy of Dr P. John).

the dark, thereby emphasizing their essential similarity to mitochondria in this respect.

The ATPase (CF_1) has been isolated from chloroplasts and shown to resemble closely the coupling factor from mitochondria. Essentially, this coupling

factor catalyses an electrogenic exchange between protons and ADP and P_i. If the chloroplasts are illuminated in the presence of ^3H_2O but in the absence of P_i and ADP, and the coupling factor is subsequently isolated from the chloroplasts, the purified protein is found to be labelled with ^3H (Ryrie & Jagendorf 1972). In the presence of ADP and P_i less label remains attached to the coupling factor after isolation. If the experiment is done in the presence of uncouplers of photophosphorylation no label is detected in the coupling factor protein. This result is consistent with the suggestion that when it catalyses the formation of ATP the protein undergoes a conformational change which is detected by the incorporation of label in the coupling factor protein. Where uncouplers are present, the intermediate labelled state is discharged because the conformational change is no longer maintained.

No attempt has been made in this résumé to give detailed and comprehensive coverage to chloroplast metabolism. Rather, I thought it more useful to give brief examples of some of the experimental approaches that have led to current views on the energy-transducing activity of illuminated chloroplasts. This list of approaches is by no means exhaustive and several intermediates have been intentionally ignored, since they have been investigated by techniques already mentioned in connection with another intermediate; moreover, I have not considered inhibitors of the system or the use of mutants, though much information has been obtained by their use. I hope, however, that I have given a sufficient indication of the basic principles underlying the energetic biochemistry of the chloroplast.

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Discussion of the three preceding papers

Hess: The existence of promitochondria in anaerobic yeast seems to indicate an intermediary state in the development of obligatory aerobic eukaryotes from anaerobic eukaryotes, which contain promitochondria and were, during evolution, generated by incorporation of anaerobic bacteria by host cells. Later, oxygen converted promitochondria into mitochondria and obligatory eukaryotes appeared. Thus, the promitochondria might have a significant function in the evolutionary concept of the development of energy transforming systems. We should remember that Slonimski observed the generation of promitochondria when yeast is maintained in the absence of oxygen. Promitochondria are free of cytochromes. We now know that they contain a membrane-bound ATPase (which is oligomycin-sensitive), bound ATP, ADP and FAD. They retain osmotic activity and the property of controlled cation permeation. However, their physiological function is still obscure.

Lipmann: Oxygen converts mitochondria into respirating organelles just as light drives the synthesis of chlorophyll. Loss and gain of respiration or photosynthesis are reversible processes (Luzikov *et al.* 1971). This, I consider, indicates that somehow oxygen induces respiration and light induces photosynthesis, but not that the uninduced organelle is a precursor of the induced.

Klingenberg: The energy potential of the ATP in the mitochondria is considerably lower than in the cytosol. So, a symbiosis of this originally prokaryotic system with the cytosol had to develop in the eukaryotic cell a system in which the intra- and extra-mitochondrial ATP potential could coexist; this amounts to the active transport which I shall describe (pp. 105–120). The redox potential gradient in the respiratory chain used to generate ATP may not be great enough for the ATP potential in equilibrium with glycolysis. Thus, in the cytosol of eukaryotic cells, ATP is required with a higher potential than in the aerobic prokaryotic system, which had to adapt to the supply of energy

from electron transport. A real problem exists here: the transport chain does not provide enough energy to maintain an ATP potential at the level required for the glycolytic system. The difference has to be made up by the 'active' transport system.

The relation between the redox potential of the NAD system and the ATP potential is the opposite in the cytosol of that in mitochondria: the increase in the redox potential of NAD is coupled to an increase in the phosphorylation potential of the ATP by the glycolytic phosphorylation. For the mitochondria, the opposite relation holds: when one decreases the redox potential of the NAD system, the phosphorylation potential increases.

Lipmann: There is some reluctance to approve the arguments for the prokaryotic origin of mitochondria while the descendance of chloroplasts from prokaryotic blue-green algae is more easily accepted. When I discussed with François Jacob the related problem of lack of true evolution in prokaryotes, he hesitated at first but then remarked that prokaryotes don't die. He considers a cycle of life and death as essential for evolution.

Whatley: May I tell an evolutionary fairy-story? *Pelomyxa palustris*, a primitive amoeba without mitochondria, has an anaerobic metabolism that basically generates lactic acid as its end product. If the organism is kept anaerobic it cannot survice because lactic acid is toxic for it; so although it has essentially an anaerobic metabolism, it demands oxygen. *Pelomyxa* carries with it its own supply of aerobic bacteria encapsulated in small vacuoles, which mop up the unwanted lactic acid by using it aerobically as an energy source for their own use. So, here is a protoeukaryote containing a bacterium, which though not making ATP for the host organism, removes the unwanted lactic acid; thus, it represents an early symbiosis in evolution. The next evolutionary step is the incorporation of the prokaryotic organism (which is an aerobic organism) into the general metabolism of the protoeukaryote by the development of a permease system, so that the ATP made by the aerobic prokaryotic organism can now enter the metabolism of the protoeukaryote, *Pelomyxa* in this example. This gives a proper eukaryotic organism of the sort we are accustomed to (John & Whatley 1976) with mitochondria.

Hagins: Professor Lipmann, what is the ordinate on your plot of evolution (Fig. 13)?

Lipmann: It is meant to be the rate of evolutionary progress, but it seems rather difficult to define units for measuring the rate of evolution.

Huxley: We might measure the rate of evolution in Darwins!

Lipmann: The graph obviously implies an accelerated progress in the last sector. I am uneasy about proposing a scale for an evolutionary rate of progress

expressible in numerical units. If so, Professor Huxley's suggestion is most welcome.

Glynn: Professor Klingenberg, you said that mitochondria will transport ions if you add ionophores but not without. Do they not transport calcium ions even without the addition of ionophores?

Klingenberg: They do. This calcium transport, however, is a physiologically important process, for example, in muscle relaxation. It probably cannot compete with the cytoplasmic reticulum.

Professor Whatley, does the chloride effect indicate that some active ion transport is tapped and is this related to the increased formation of the phosphorylated intermediate in CO_2 fixation?

Whatley: Chloride does not specifically tap active ion transport. Our experiments (Fig. 6) demonstrated that the ability to make reduced pyridine nucleotide is lost when chloride ions are removed. Consequently, the initial product of CO_2 fixation can no longer be reduced. Cyclic phosphorylation operates perfectly well in the absence of chloride ions, so that light energy is transduced into ATP, but ATP on its own is of no value for CO_2 fixation; the reductant NADPH and ATP are both necessary. The mechanism by which chloride ions operate is still unknown. Chloride might act as a 'balancing ion'. For example, in a redox system such as $Mn^{2+} \rightarrow Mn^{4+}$, in which the manganese is in a chelated form, it may be necessary for chloride ions to enter the system to provide electrochemical balancing and the stabilization of the intermediate before the redox process can continue to cycle. Thus, in the absence of chloride, oxygen evolution and therefore NADP reduction may not be possible.

Keynes: How specific is the requirement for chloride ions?

Whatley: Bromide ions can be substituted *in vitro* but not physiologically for chloride ions. Thiocyanate works to some extent but nitrate is much less active; phosphate, sulphate and acetate ions are ineffective. The chloroplast system is then essentially specific for chloride.

Roseman: Is the chloroplast freely permeable to chloride ions?

Whatley: In the dark, chloride ions will not enter the chloroplast; chloroplasts have no cytochrome oxidase and, therefore, no aerobic metabolism, so no active transport system is available. However, in the light an active transport of chloride can occur, driven by the light-driven proton flux.

Roseman: What is the stoichiometry of the 'acid-bath' experiment? I ask because we do have some idea of how many protons move out during ATP synthesis.

Whatley: The stoichiometry appears to be $2H^+$/ATP (Carmeli 1970). When the experiment was first performed, it was asked whether the formation of ATP could not be explained by a back up of electrons (reverse electron flow)

through the electron transport chain. In view of the number of molecules acting in the chain and the electrons that can be loaded onto them during the first part of the acid-bath experiment ('pickling in acid'), reversed electron flow is theoretically possible. It is, however, certain that the amount of ATP formed is much in excess of the equivalent reverse electron flow that is possible. The accumulation of protons inside the thylakoid is much greater and corresponds to about two protons/ATP synthesized. It is as if there were a pump operating electrogenically to make the ATP.

Roseman: Both phosphate and ADP enter freely?

Whatley: Essentially, yes. The chloroplast particles that perform photoreactions are 'inside out' with respect to mitochondria (Fig. 14). When one isolates a chloroplast, it is experimentally difficult to keep the outer membrane intact.

All the observations of photochemical reactions are made with 'naked lamellar systems' or broken chloroplasts which have lost the outer membrane. ATP, ADP and phosphate, which could not normally pass the outer boundary membrane, are then completely accessible to the thylakoid membranes, which are equivalent to submitochondrial particles, in terms both of their direction of proton pumping and in the location of the coupling factor, ATPase. In the intact chloroplast ATP, ADP and phosphate are mainly exchanged across the boundary membrane indirectly through the use of triosephosphates and PGA.

Glynn: Some time ago, MacRobbie (1965) showed that giant algal cells could pump chloride ions across the plasmalemma using light energy in a way that bypassed ATP. Can chloroplasts move chloride ions across their membranes or is this something peculiar to algae?

Whatley: The processes may be analogous and may include chloride transport across a membrane as a result of proton pumping. In the algal cell (as with most differentiated plant cells), a large central vacuole is bounded by the tonoplast membrane, which in many ways resembles the outer membrane of an animal cell. In a sense, the vacuole has the same relation to the plant cell as the external environment has to the animal cell. Chloride is excreted into the vacuole against a gradient and the process requires energy, which can be supplied either aerobically in the dark or anaerobically in the light. The chloroplast can only take up chloride in the light since it has no anaerobic metabolism of its own.

Wilkie: Are there any circumstances under which chloroplasts break down ATP to ADP and P_i? I ask because I am interested in reversible systems in which the hydrolysis of ATP is driven by ions whose activity can be measured directly, thereby giving us a means for directly measuring the change in free energy of ATP hydrolysis.

Whatley: Curiously, chloroplasts generally have extremely low (almost negligible) ATPase activity unless they are vigorously stimulated as, for instance, by being illuminated in the presence of a strong reducing agent, like cysteine or dithiothreitol (Petrack & Lipmann 1961). Under these circumstances, the coupling factor which is normally buried in the membrane appears to become uncovered so that the ATPase activity can be observed. I don't know the answer to the question in terms of specific movement of ions but, presumably, from the Mitchell hypothesis, ATP hydrolysis or synthesis depends on a countermovement of protons.

Lipmann: In our experiments on the photohydrolysis of ATP in chloroplasts in the presence of thiolic compounds (Petrack & Lipmann 1961; Petrack *et al.* 1965), we found that by increasing the concentration of cysteine or, better still, thioctic acid [5-(1,2-dithiolan-3-yl)valeric acid], we could almost titrate the transition from a photophosphorylation to a photohydrolysis. The photohydrolysis of ATP persists after irradiation; it decays within 50 s (Petrack *et al.* 1965).

Hastings: Many organic molecules are suitable for the study of the transfer of energy from one molecule to another. The fact that such energy transfer among identical molecules fails to occur at the red-edge of the absorption band has been suggested as an explanation (Weber & Shinitzky 1970) of Emerson's results (Emerson *et al.* 1957) where energy transfer in the chlorophyll of the antenna presumably acts through identical molecular species. Has this suggestion gained any support?

Whatley: I don't think so. However, these experiments, being physiological, are somewhat complex: there may be more contributions to the total effect than Emerson's red-drop. Originally, Emerson *et al.* (1957) stated that they considered the possibility that their results implied two light reactions in photosynthesis but rejected this interpretation. Here, Emerson's experimental results are being interpreted to demonstrate the occurrence of two light reactions. Enhancement indicates not that light at, say, 708 nm is lost to the system by an edge effect, but that the system is simply unable to use the light absorbed owing to the absence of a second wavelength of light to drive the second of the two coupled photoreactions. The energy absorbed at 708 nm is not, therefore, stored as chemical energy but can be lost as fluorescence or as heat when the shorter wavelength is missing.

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Energy changes during the formation and interconversion of enzyme–substrate complexes

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Abstract The rate constants and equilibrium constants of the individual steps of several enzyme reactions may be determined by the application of rapid reaction methods and isotope techniques. This makes it possible to complement the formalism of the Haldane relation with details of the reaction mechanism. It has been shown that, in several enzyme reactions, steps involving chemical catalysis are fast and have small free-energy changes compared with those of the substrate binding and product dissociation processes. Data are presented in this paper for three enzyme reactions for which different methods have been used to elucidate the kinetic parameters of the elementary steps.

For cardiac lactate dehydrogenase (EC 1.1.1.27), absorption and fluorescence spectroscopy have been used to distinguish the step involved in the chemical process from those involved in the formation of the substrate complex and the release of the product. The rate of interconversion between enzyme-bound substrates and products is fast compared with other steps and the equilibrium constant for the process is near unity. Consequently, the difference of standard free energy changes for the formation of the two ternary complexes corresponds approximately to the overall free-energy change of the hydrogen transfer reaction.

Isotope kinetic techniques can be used to study the reactions of triosephosphate isomerase (EC 5.3.1.1). With this enzyme, the interconversion of enzyme-bound substrate into product is comparable in rate to product dissociation.

The reactions of myosin subfragment 1 with ATP, studied by fluorescence spectroscopy and chemical quenching, follow a similar pattern in that the equilibrium constant of the chemical step in which water reacts with protein-bound ATP is 9. In this case, however, there is a remarkably large free-energy change associated with a first-order process involved in the binding of ATP. The possible significance of these results to energy transduction in muscle contraction as well as in the biosynthesis of ATP is discussed.

In his classical treatment of steady-state kinetics of enzyme reactions, Haldane (1930) derived equations relating reaction rates of reversible processes to the overall equilibrium. For the simplest possible scheme (1), in which the free

enzyme E reacts with the free substrate S to give the product P, he showed that

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{EP} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{E} + \mathrm{P} \tag{1}$$

the overall equilibrium constant, K, is given by (2), where [P] and [S] are the

$$K = [\mathrm{P}]/[\mathrm{S}] = k_{+1}k_{+2}k_{+3}/k_{-1}k_{-2}k_{-3} \tag{2}$$

equilibrium concentrations of free product and substrate, respectively and k_i is the rate constant for the ith step. When [P] is the concentration of total product, this equilibrium relation is restricted to the particular case when the enzyme concentration is negligible compared with the other reactants—a condition which often does not hold in biological systems. This expression (2) is equivalent to the statement that for a series of sequential reactions the overall standard free-energy change is the sum of the standard free-energy changes of each individual step. Consequently, the overall equilibrium constant is the product of the equilibrium constants for the individual steps. The dissociation constant for the substrate, K_S, is k_{-1}/k_{+1} and the dissociation constant for the product, K_P, is k_{+3}/k_{-3}. It follows that the overall equilibrium for this simple scheme (1) is given by (3) and that the equilibrium constant for the chemical inter-

$$K = \frac{K_P}{K_S} \cdot \frac{k_{+2}}{k_{-2}} \tag{3}$$

conversion, k_{+2}/k_{-2}, is related to the overall equilibrium constant by the ratio of product affinity to substrate affinity. Here we are concerned with those aspects of information about reactions catalysed by enzymes obtained in the period since Haldane's postulates were derived. These give more insight into equilibria of reactions within the enzyme complexes.

The application of rapid-reaction techniques to the direct observation of the formation and decomposition of intermediates in enzyme reactions, begun by Chance in the late 1930s (see Chance 1951), was greatly extended during the last 25 years (Gutfreund 1971). From the point of view of the present discussion the two general results obtained which modify one's thinking about the Haldane relation, although they do not affect the principle, are the following. First, the combination of enzyme with substrate in most enzyme reactions is not adequately represented by a single step. The initial second-order association is usually followed by an isomerization. The second-order rate constants so far determined for such reactions have been found to be remarkably invariant (in the range 10^7–10^8 l mol^{-1} s^{-1}) and close to the maximum possible for

diffusion controlled reactions, although rapid partitioning between reactive and unreactive forms (e.g. specific protonated states of enzyme or substrate) can give an apparently low value for the second-order rate constant. The specific processes—the reactions which appear to differentiate in the energy of binding of substrates and products—are the isomerizations within the initial complex. The second revelation which has a bearing on the present discussion is the fact that chemical catalysis (second step in scheme [1]) is not usually rate limiting for the overall reaction. In many of the reactions studied in detail (see Gutfreund 1971), the rate-limiting step for the overall reaction is either the isomerization in the formation of the reactive complex or some step in the dissociation of products. The distinction between binding energies of substrate and of product is often expressed as the difference in their rate of dissociation. The three examples we shall describe in some detail illustrate how the identification of intermediates and the kinetic analysis of interconversion can be used to deduce the energy relations between the different complexes. The transient kinetic analysis and the spectroscopic and chemical identification of the intermediates of the reactions of lactate dehydrogenase (EC 1.1.1.27) and of myosin demonstrate the general principles governing the properties of the large groups of reactions involving hydrogen transfer and hydrolysis. Another approach is illustrated with the results obtained by Knowles & Albery (unpublished results) on triosephosphate isomerase (EC 5.3.1.1).

THE IDENTIFICATION OF TRANSIENT INTERMEDIATES OF LACTATE DEHYDROGENASE

Transient kinetic techniques enable us, first, to determine the number of intermediates in the reaction pathway and the time scale on which they are formed and interconverted. Secondly, we can apply the monitoring methods (spectroscopy, etc.) used to follow the reactions on the time scale of a single turnover of (enzyme + substrate) into (enzyme + product) to identify the nature of the intermediates and the chemical or physical changes which occur during their interconversion.

We shall be concerned with flow techniques with a time resolution of about 1 ms, with absorption and fluorescence spectroscopy and with chemical quenching for the observation of the reactions.

Details of our experiments on cardiac lactate dehydrogenase have recently been described (Holbrook & Gutfreund 1973; Whitaker *et al.* 1974). We shall summarize the evidence for those features of the following reaction scheme (4) which are significant here (only the different forms of the enzyme are included in the scheme). Steps 2 and 4 are likely to include isomerizations of the ternary

$$E \overset{1}{\rightleftharpoons} ENAD^+ \overset{2}{\rightleftharpoons} E^{NAD^+}_{Lactate} \overset{3}{\rightleftharpoons} H^+E^{NADH}_{Pyruvate} \overset{4}{\rightleftharpoons} ENADH \overset{5}{\rightleftharpoons} E \qquad (4)$$

complexes as well as the simple association–dissociation. Kinetic evidence, summarized by Holbrook & Gutfreund (1973), indicates that such an isomerization in step 4 is rate limiting for the reaction in both directions. Ordered addition with the nucleotide associating first and dissociating last was established from steady-state investigations (Schwert *et al.* 1967).

The analysis of stopped-flow experiments with lactate dehydrogenase from pig heart by absorption and fluorescence spectroscopy allowed the identification of two distinct steps (3 and 4) which controlled the rate of formation of the enzyme product (Fig. 1). When enzyme is mixed with excess NAD^+ and lactate, steps 1 and 2 are much faster than any of the subsequent reactions. At pH 7, the observation, from the extinction changes at 340 nm, of NADH being formed provides evidence for three distinct rates. First, there is a fast phase ($t_{\frac{1}{2}} < 1$ ms) during which the amount of NADH formed corresponds to 0.2 equivalents of the total enzyme sites in the solution. This is followed by NADH formation corresponding to 0.8 equivalents of the enzyme sites with a half time $t_{\frac{1}{2}} = 5$ ms. Finally, NADH is formed at the steady-state rate of the enzyme turnover. Analysis of the same reaction by protein fluorescence and nucleotide fluorescence indicates that the fastest phase corresponds to the formation of NADH in the ternary complex with enzyme and pyruvate, $H^+E^{NADH}_{Pyruvate}$. Observation of free H^+ formation (in the presence of indicators) shows that no protons are liberated during that phase. During the next period of NADH production ($t_{\frac{1}{2}} = 5$ ms), fluorescence monitoring indicates the formation of ENADH with release of pyruvate and monitoring of pH indicates the liberation of protons at the same rate.

We have concluded from this evidence that the rapid production of NADH during the formation of the ternary product complex corresponds to the rapid equilibration indicated as step 3 in scheme (4). The equilibrium constant K at pH 7 is 0.25 (Eq. 5),

$$K = [H^+E^{NADH}_{Pyruvate}]/[E^{NAD^+}_{Lactate}] = 0.25 \qquad (5)$$

whereas the equilibrium constant for the overall reaction at pH 7 is given by (6).

$$K = \frac{[\text{Pyruvate}]\ [\text{NADH}]}{[\text{Lactate}]\ [NAD^+]} = 0.37 \times 10^{-4} \qquad (6)$$

These results show that the chemical steps of the enzyme catalysed reaction are fast compared with product dissociation and that the free-energy change during the interconversion of ternary complexes is small. The difference

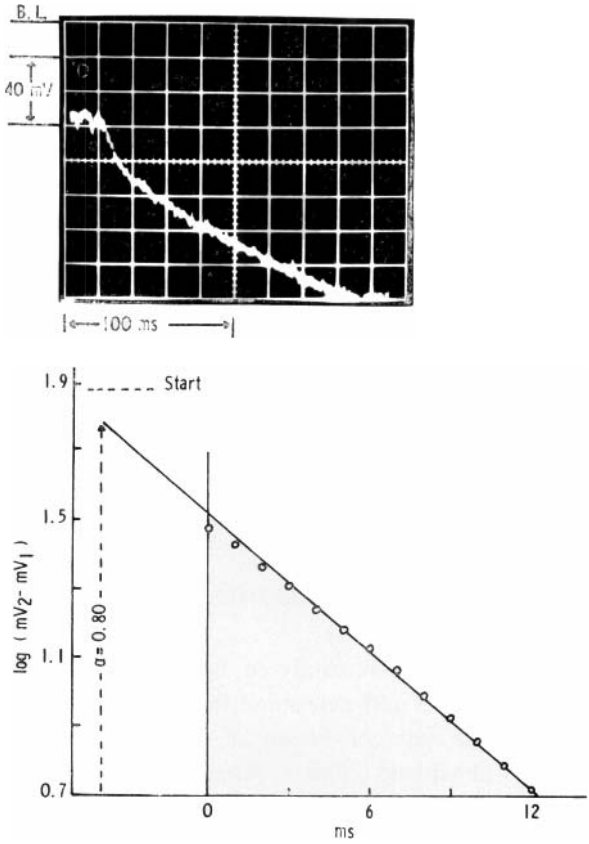

FIG. 1. Record of a stopped-flow experiment for the measurement of absorbancy (1 volt corresponds to $\varepsilon_{340}^{1\,cm} = 1$) at 340 nm of the reaction of lactate dehydrogenase from pig heart (15 μmol/l in sites) with 1.34mM-NAD^+ and 100mM-lactate in phosphate buffer at pH 7.

The base line (B.L.) corresponds to zero extinction difference between reactant solutions and mixture. The first-order plot of the approach to the steady state is extrapolated back 4 ms to account for the age of the mixture when it first reaches the observation chamber. The difference between the extinction at this extrapolated zero time of the transient (corresponding to reaction step 4 in eqn. [4]) and the base line represents the amplitude of the much faster reaction identified as step 3. From this extinction difference we find that the fraction of enzyme sites containing bound NADH within 1 ms of mixing equals 0.20 ($= 1 - \alpha$).

between the equilibrium constants in (5) and (6) must be compensated by the differences between the free-energy changes of enzyme–substrate and enzyme–product complex formation. This is borne out by the considerably greater stability of the complex $H^+E^{NADH}_{Pyruvate}$ as compared with the stability of $E^{NAD^+}_{Lactate}$.

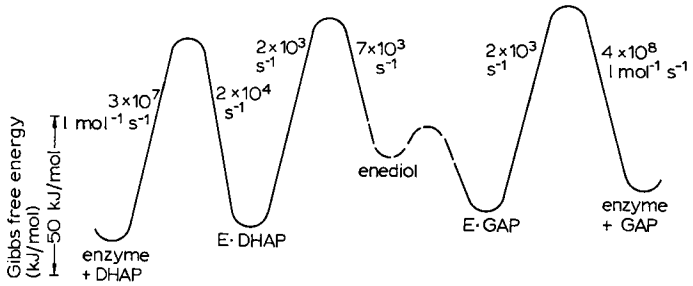

FIG. 2. Free-energy profile for the reaction catalysed by triosephosphate isomerase: ——, experimentally quantified parts of the reaction scheme; – – – –, parts that are currently inaccessible. The profile drawn relates to that in which the overall equilibrium constant $K = 420$ for the unhydrated forms of the substrates, D-glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). This fixes the free-energy difference between enzyme (E) + GAP and enzyme + DHAP. The free-energy difference between E + DHAP and E·DHAP depends on the definition of the standard-state concentration of free unhydrated DHAP. This is taken to be the same as the *in vivo* concentration of DHAP (40 μmol/l) and not 1 mol/l. Rate constants to the left of the peaks are associated with processes in the conversion of DHAP into GAP, and to the right of the peaks with the reverse reaction.

ISOTOPE KINETIC STUDIES OF TRIOSEPHOSPHATE ISOMERASE
(contributed by J. R. Knowles and J. Albery)

The use of isotopic labels in the reaction catalysed by triosephosphate isomerase allows one to trace the course and determine the rates of proton transfer reactions that constitute the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. This approach is described by Knowles *et al.* (1971).

Four interdependent approaches have been taken: (*a*) 'in-exchange:conversion' experiments in which the appearance of ^3H in the substrate and in the product of the reaction is followed as a function of the extent of the reaction in ^3H_2O; (*b*) 'transfer:out-exchange' experiments in which the fate of a stereospecific tritium atom in substrate is followed, and its distribution between remaining substrate, product and solvent is determined after incomplete reaction; (*c*) deuterium isotope effect studies in which the effect of specific deuteriation of substrates on the steady-state rates of the catalysed reaction is determined; and (*d*) measurement of the steady-state rates of reaction of the all-hydrogen system.

From the results of these experiments, Knowles & Albery have been able to describe completely the energetics of the reaction catalysed by triosephosphate isomerase. The free-energy profile (Fig. 2) is remarkable in that it appears that this enzyme has reached perfection in evolutionary terms, and that further erosion of any transition state or destabilization of any intermediate would be

unprofitable. This is because the extent to which any catalyst can speed up a reaction is limited by (i) the thermodynamics of the situation and (ii) the diffusion rate for substrate finding catalyst. With lactate dehydrogenase, the catalysed change of covalent bonds proceeds at a rate that is much faster than the steps dissociating the product. With the isomerase, the rates are comparable.

THE IDENTIFICATION OF TRANSIENT INTERMEDIATES OF MYOSIN ATPase

We have so far considered the standard free-energy changes of elementary steps of two enzyme catalysed reactions. These enzymes are not strictly linked in biology to energy transduction processes. In contrast, the myosin and actomyosin ATPases are likely to be intimately linked with energy transduction in muscle. We now consider the equilibrium constants and hence standard free-energy changes accompanying steps of the catalysis.

The mechanism of the Mg^{2+}-dependent myosin ATPase isolated from rabbit skeletal muscle has been studied principally with heavy meromyosin and subfragment 1, products of mild proteolytic digestion of myosin (Lowey *et al.* 1969). Both proteins retain the ATPase activity and the actin-binding site of myosin. Heavy meromyosin is a dimeric protein of molecular weight 340 000 containing both 'heads' of the myosin molecule whereas subfragment 1 is monomeric with molecular weight 120 000. These proteolytic fragments have the advantage over myosin for kinetic studies of being soluble at physiological ionic strength.

Since ATP binds relatively rapidly and tightly to subfragment 1, M, and the rate-limiting step of the Mg^{2+}-dependent ATPase is breakdown of a complex between subfragment 1 and products, $M^{**}{\cdot}ADP{\cdot}P_i$, we can measure the equilibrium constant of the cleavage of ATP as follows. We mixed ATP with a molar excess of subfragment 1 and then stopped the reaction by quenching in perchloric acid after the binding of ATP to the protein was complete but before the myosin–products complex had had time to break down. The reaction whose equilibrium constant we can measure is represented by equation (7).

$$M^{*}\cdot ATP \rightleftharpoons M^{**}\cdot ADP\cdot P_i \qquad (7)$$

The asterisks are needed (see later) to distinguish intermediates of the mechanism but they also serve the useful function of indicating that the intermediate concerned has enhanced fluorescence relative to subfragment 1. (A single asterisk represents about a 10% enhancement and two asterisks 20%.)

The time course of the protein fluorescence during a single turnover experiment (Fig. 3) is used to indicate the appropriate time after mixing to quench

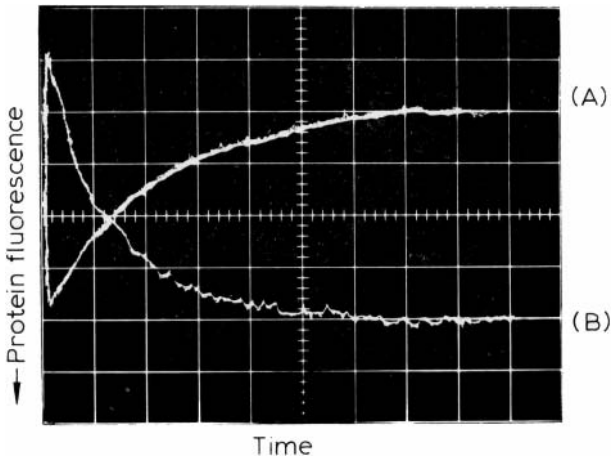

FIG. 3. Stopped-flow spectrophotometric record of protein fluorescence during a single turnover of the subfragment 1 ATPase at 21 °C. One syringe contained subfragment 1 (15 μmol/l; reaction chamber concentrations) and the other 2.5μM-ATP. Both syringes contained 5mM-$MgCl_2$–50mM-KCl–20mM-Tris buffer adjusted to pH 8.0 with HCl. The protein was excited by light at 300 nm and the emitted light between 335 and 375 nm was analysed. For (A), the time scale is 5 s/division and for (B) it is 50 ms/division.

the reaction. In particular, it gives a value for τ, the time at which the binding of ATP to the protein is more than 99% complete. In the reaction shown in Fig. 3, τ is 300 ms (from trace B), since at this time the fluorescence enhancement characteristic of the binding and cleavage process has reached a maximum. In practice, the technique used in these experiments was such that the first time after τ that quenching could be performed was two seconds. For this reason, the slow phase (Fig. 3A) contains important information since it demonstrates the rate at which $M^{**}\cdot ADP\cdot P_i$ has decayed, and hence the fraction of nucleotide present as $M^{*}\cdot ATP + M^{**}\cdot ADP\cdot P_i$ at any time can be calculated. Table 1 shows the amount of ATP present at various times during the reaction. Most important is the fact that at 2 and 5 s (both times $> \tau$), some ATP is present and is decaying at the same rate as protein fluorescence. From this exponential decay rate (0.10 s^{-1}) and the concentration of ATP at 2 s, the concentration of ATP can be calculated at time τ by extrapolation; it equals 10% of the total nucleotide. It follows that the equilibrium constant K is given by (8).

$$K = [M^{**}\cdot ADP\cdot P_i]/[M^{*}\cdot ATP] = 9 \qquad (8)$$

This experiment and the control experiments showing that all the nucleotide

TABLE 1

Concentration of M*·ATP during a single turnover of subfragment 1 ATPase

Quenching time (s)	*Amount of [^{32}P]ATP present (%)*
0	100
2.0	7
5.0	4
60	0.3

The experiment was done at 21 °C with 15μM-subfragment 1 (reaction chamber concentrations) and 2.5μM-[γ-^{32}P]ATP in the same solvent as described in the legend to Fig. 3. The mixing of the reagents and the analysis of the products are described by Bagshaw & Trentham (1973). Each value is an average of duplicate assays which agreed within 2% and has been corrected for the decomposition of ATP during quenching which was 4% of the ATP present at the time of quenching.

is tightly bound to the protein at time τ are described by Bagshaw & Trentham (1973).

Chemically, experiments on incorporation of ^{18}O suggest that water has reacted with ATP in step 3 in eq. (9) (Sartorelli *et al.* 1966). However, the quenching technique does not allow us to distinguish whether M**·ADP·P_i reflects the true hydrolytic products bound to the enzyme or an adduct between ATP and H_2O such as might occur with the involvement of a pentacoordinate γ-P atom (Bagshaw & Trentham 1973). It is, nevertheless, convenient to refer to M*·ATP $\rightleftharpoons$ M**·ADP·P_i as the cleavage step.

A two-step binding mechanism for ATP to subfragment 1 has been proposed on the basis of several experimental observations (Bagshaw *et al.* 1974). One example will suffice. The kinetics of binding of ATP(γS) (adenosine 5′-[3-thiotriphosphate]) and of ATP to subfragment 1, as reflected by protein fluorescence changes, are indistinguishable. In contrast to ATP, however, ATP(γS) is cleaved slowly (0.25 s^{-1} at pH 8 and 21 °C) so that M*·ATP(γS) is the steady-state complex of ATP(γS) hydrolysis catalysed by subfragment 1. It follows that the rates of the rapid changes in protein fluorescence (illustrated for example by Fig. 3) are controlled by the binding process. If the binding of ATP(γS) to subfragment 1 was a simple bimolecular process, then the observed rate of the fluorescence change, k_{obs}, should increase linearly with [ATP(γS)] (for [ATP(γS)] $>$ [subfragment 1]). This is not so (Fig. 4). A two-step binding mechanism is, however, compatible with the data; the initial formation of a loosely associated complex is followed by a first-order isomerization. The equilibrium constant, K_1, and rate constant k_{+2} (see equation 9) can be obtained from such experiments when ATP is used.

Experiments such as those described (see also Lymn & Taylor 1970) together

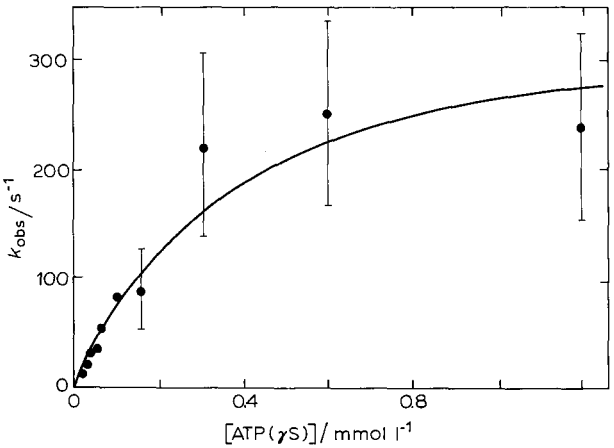

FIG. 4. Kinetic analysis of the fluorescence enhancement of subfragment 1 on interaction with ATP(γS): k_{obs} equals the rate of fluorescence increase analysed as an exponential process when ATP (γ S) was mixed with 5.0μM-subfragment 1 in a medium of 0.1M-KCl–5mM-$MgCl_2$–50mM-Tris adjusted to pH 8.0 with HCl. The non-linear relation between k_{obs} and [ATP-(γS)] establishes that a simple second-order binding process does not adequately describe the reaction. The solid line describes a hyperbolic function that is consistent with a two-step binding process. The experimental method and data analysis are described by Bagshaw *et al.* (1974).

with an elegant series of experiments by Mannherz *et al.* (1974), which demonstrated the formation of protein-bound ATP when ADP was mixed with a molar excess of subfragment 1 in the presence of P_i, have allowed a mechanism to be formulated for the Mg^{2+}-dependent ATPase of subfragment 1. All the equilibrium constants and many of the rate constants are known (equation 9).

$$\begin{aligned} M + ATP &\overset{K_1}{\rightleftharpoons} M{\cdot}ATP \overset{K_2}{\rightleftharpoons} M^*{\cdot}ATP \overset{K_3}{\rightleftharpoons} M^{**}{\cdot}ADP{\cdot}P_i \overset{K_4}{\rightleftharpoons} M^*{\cdot}ADP{\cdot}P_i \\ &\overset{K_5}{\rightleftharpoons} M^*{\cdot}ADP + P_i \overset{K_6}{\rightleftharpoons} M{\cdot}ADP \overset{K_7}{\rightleftharpoons} M + ADP \end{aligned} \qquad (9)$$

Structural and kinetic evidence exists for intermediates $M^*{\cdot}ATP$, $M^{**}{\cdot}ADP{\cdot}P_i$ and $M^*{\cdot}ADP$. $M{\cdot}ATP$ and $M{\cdot}ADP$ are characterized on kinetic grounds (Bagshaw *et al.* 1974). The strongest evidence for $M^*{\cdot}ADP{\cdot}P_i$ comes from the observation (Mannherz *et al.* 1974) that P_i saturates $M^*{\cdot}ADP$ in the experiments on the formation of $M^*{\cdot}ATP$. Values for the equilibrium constants at 21 °C in a medium of 0.1M-KCl–5mM-$MgCl_2$–50mM-Tris adjusted to pH 8.0 with HCl are, according to this mechanism (equation 9):

$K_1 = 4.5 \times 10^3\,l\,mol^{-1}$; $K_2 = 3.7 \times 10^9$; $K_3 = 9$; $K_4 = 15$; $K_5 = 8 \times 10^{-3}$

mol l^{-1}; $K_6 = 3.5 \times 10^{-3}$; $K_7 = 2.7 \times 10^{-4}$ mol l^{-1} and values for the rate constants are $k_{+2} = 400\ s^{-1}$; $k_{-2} = 1.1 \times 10^{-7}\ s^{-1}$; $k_{+4} = 6 \times 10^{-2}\ s^{-1}$; $k_{-4} = 4 \times 10^{-3}\ s^{-1}$; $k_{+5} = 1.4\ s^{-1}$; $k_{-6} = 400\ s^{-1}$. Both k_{+1} and k_{-7} are $>$ 10^7 l mol^{-1} s^{-1}, $k_{+3} \geqslant 160\ s^{-1}$ and $k_{+5} > 50\ s^{-1}$.

The value of K_2 ,which is central to the theme of this paper, is deduced from a knowledge of all the other equilibrium constants and the overall equilibrium constant, $K = [ADP][P_i]/[ATP] = 1.7 \times 10^7$ mol l^{-1} in these conditions (Alberty 1969). However, the determination of K_2 is not so indirect as may appear at first sight because the experiments of Mannherz *et al.* (1974) directly compare the overall equilibrium constant of ATP binding ($= K_1K_2$) with the product of the remaining equilibrium constants ($= K_3K_4K_5K_6K_7$). Moreover, the formation of protein-bound ATP that they observe occurs at just the predicted rate which is essentially the catalytic centre activity of the ATPase which has a well characterized value. This kinetic consistency reinforces our confidence that they are observing genuine formation of M*·ATP.

The high ratio of K_2 to K_3 is striking, as is the high affinity of ATP for subfragment 1. Moreover comparison with the free-energy profile of triosephosphate isomerase illustrates that, whereas in that mechanism the difference between adjacent free-energy troughs is no greater than 5 kJ mol^{-1} (the enediol trough is indeterminate), in the case of subfragment 1 the free-energy difference associated with the first-order isomerization on ATP binding is 44 kJ mol^{-1}.

RELATIONSHIPS BETWEEN ENZYME CATALYSIS AND ENERGY TRANSDUCTION

The mechanism of the ATPase deduced from such solution studies probably provides a good model for the ATPase of myosin in relaxed muscle. To understand energy transduction in muscle from the biochemical viewpoint, we must analyse the actomyosin ATPase in an organized system (such as a muscle fibre) which is capable of doing mechanical work. At best, solution studies of the actomyosin ATPase provide a model for actively contracting muscle under zero load.

An important feature of the model of Lymn & Taylor (1971) which relates the biochemical events of the actomyosin ATPase with the cross-bridge cycle of actively contracting muscle is that the cleavage step in the actomyosin ATPase mechanism occurs when actin and myosin are detached. The cleavage step is then common to both myosin and actomyosin ATPase mechanisms. If their model is basically correct, it is desirable, though not essential, for there to be a relatively small standard free-energy change in the cleavage step (Bagshaw

& Trentham 1973). The fact that this appears to be so strengthens the case for the Lymn–Taylor model.

Much work remains to be done to clarify the biochemical events associated with energy transduction in muscle because the intermediates and equilibrium constants of individual steps of the actomyosin ATPase, both in solution and in more organized heterogeneous systems, have yet to be characterized. New techniques must be developed to measure the concentrations of these intermediates in actively contracting muscle before the changes in free energy of individual steps can be determined. Such further work will illustrate the significance of the large negative free-energy change of the isomerization of the myosin ATP complex described above. Recently, Taylor (1973), Cooper (1973) and Hill (1974) have detailed methods to describe energy transduction in muscle from the viewpoints of thermodynamics and statistical mechanics.

Boyer *et al.* (1974), who have carried out a wide range of isotope exchange studies on ATPases, have noted striking analogies between the mechanisms of the myosin ATPase and of proteins catalysing ATP formation such as the mitochondrial ATP synthase. In this synthase, tightly bound ATP is readily synthesized from ADP and P_i but input of energy is required to release ATP into the medium. Of the two alternatives suggested by Boyer *et al.* (1974) for coupling the energy derived from mitochondrial oxidative processes, the one supported by Mitchell (1974) is perhaps the most attractive; namely, a conformational change (analogous to $M^*{\cdot}ATP \rightleftharpoons M{\cdot}ATP$), if present, is facilitated by a membrane potential and thus this potential drives ATP release. Direct evidence that membrane potentials in either mitochondria, chloroplasts or bacteria can promote this conformational change of the appropriate ATP synthase and hence ATP release is required. It is not difficult to envisage such a membrane potential, operating through a proton-translocation mechanism, being able to drive a conformational change of an ATP synthase. Returning for analogy to the myosin ATPase it is striking that proton uptake and release steps to the solvent accompany the isomerizations although no proton release accompanies the cleavage step, $M^*{\cdot}ATP \rightleftharpoons M^{**}{\cdot}ADP{\cdot}P_i$ (Bagshaw & Trentham 1974). This feature is also observed in the mechanism of the reaction of lactate dehydrogenase.

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Discussion

Keynes: Is it also possible to study the intermediate reactions by direct calorimetry?

Gutfreund: We are interested in this. Direct calorimetry is technically possible but will be a considerable project. Calorimetry often brings out other steps which are not detectable in spectroscopy or in fluorescence. We should like to study myosin calorimetrically, but this is difficult because of the flow properties of the muscle proteins.

Woledge: Yamada *et al.* (1973) have worked on the calorimetry of myosin; they observed the heat change when ADP binds to myosin (about 100 kJ/mol ADP bound) and similarly for the binding of ATP. They also showed a considerable heat change associated with the slow step in the hydrolysis.

Huxley: Such an amount of heat is 2–3 times the total free-energy change that we are considering for the overall reaction ATP $\rightarrow$ ADP + P_i.

Gutfreund: The changes that we want to look at in greater detail are probably much smaller. One difficulty will be the blocking out of the large heat changes of the hydrolysis. The changes we want to observe are conformational changes.

Wilkie: Are all these heat productions positive? They cannot *all* be positive.

Woledge: The heats of binding ATP and ADP are negative (exothermic). As the reactions are written, a large endothermic effect would be noticed on dissociation of ADP from myosin.

Roseman: Is aggregation or disaggregation a problem?

Gutfreund: Not in the experiments we described.

Roseman: How does this elegant kinetic study relate to what we biochemists call the Michaelis constant, K_m, of the enzyme reaction?

Gutfreund: The Michaelis constant is a combination of rate constants that depends on the mechanism. Operationally, $K_m = [S]$, the substrate concentration at half maximum velocity. That is a definition which fits any system that follows Michaelis kinetics; the velocity $v = V\,[S]/(K_m + [S])$, where V and K_m are combinations of many rate constants which depend on the particular mechanism. K_m can be smaller or larger than the substrate dissociation constant.

Roseman: In these reactions, the rate-limiting step is the final dissociation, which is not the assumption a biochemist normally makes when talking in terms of K_m.

Cohn: Does the equilibrium constant of the overall reaction with stoichiometric amounts of the enzyme differ from the value obtained with catalytic amounts of enzyme? In other words, have you considered the summation of the dissociation constants at stoichiometric amounts of enzyme and compared

it with a measured value of the overall equilibrium constant with stoichiometric amounts of enzyme?

Trentham: The high value of K_2 (p. 78) is deduced from the knowledge of the overall equilibrium constant and from the determination of the other six equilibrium constants.

Cohn: But that is the overall equilibrium constant for the reaction where myosin is catalytic and its concentration does not enter the kinetics. What is the overall equilibrium constant for stoichiometric amounts of enzyme? These remarkable changes between the overall and the individual steps are somewhat misleading because in the individual steps the amounts of enzyme are always stoichiometric.

Gutfreund: The overall equilibrium constant allowing for the enzyme has not been determined for myosin because of the difficulty of determining the amount of free ATP, but it has been determined for lactate dehydrogenase. We have calculated all the equilibrium constants separately, multiplied them together and got the right answer.

Huxley: However, for myosin ATPase, K_2 is obtained by assuming that the overall equilibrium constant is the standard figure for ATP hydrolysis and by measuring the equilibrium constants of the other intermediate steps.

Cohn: To determine K_2 by *assuming* the equilibrium constant is circular reasoning.

Trentham: One can measure the overall equilibrium constant and all the other six with the exception of K_2. From those two sets of numbers we can determine K_2.

Gutfreund: It is only circular if you don't believe in the relation that the product of the equilibrium constants for the individual steps is the overall equilibrium constant.

Roseman: With stoichiometric amounts of enzyme, intermediate forms besides the enzyme and substrate (i.e. enzyme–substrate and enzyme–product) complicate the system. For that reason, it is most important to make this direct measurement.

Trentham: It may help if I describe a typical experiment of Mannherz *et al.* (1974). The sum of the concentrations of protein-bound and free ATP in a mixture containing myosin subfragment 1 in a molar excess of ADP in, say, 10mM-P_i is measured. From the overall equilibrium constant, they know that effectively none of the ATP can be free; it must all be bound to the protein.

Cohn: Are the proportions of ATP, ADP and P_i in these experiments consistent with these constants?

Trentham: Yes.

Huxley: But isn't the concentration of free ATP so low as to be unmeasur-

able? Its value may be consistent, but can you measure it accurately enough to derive a useful check?

Taylor: Dr Cohn is asking a more embarrassing question. If the proposed scheme were wrong, the product of the equilibrium constants of the individual steps would not equal the overall figure. Measurements of all the constants would reveal the discrepancy. This information has not been obtained.

Trentham: But the experiments of Mannherz *et al.* (1974) are still useful in the sense that they measure the combined equilibrium constant of all the steps subsequent to ATP binding and, even if the scheme describing these steps is wrong in detail, they can nevertheless deduce the equilibrium constant of ATP binding (i.e. for $M + ATP \rightleftharpoons M^*{\cdot}ATP$).

It is difficult experimentally to measure free ATP (i.e. not bound to protein). Wolcott & Boyer (1974) measured the protein-bound ATP and designed an experiment to try to measure the rate of release of ATP from the protein to form free ATP. The value of k_{-2} they determined is 10^{-3} s^{-1} but the concentration of $M^*{\cdot}ATP$ is similar to that found by Mannherz *et al.* (1974). However, at this stage I do not think that the rate they observed necessarily describes the rate of ATP dissociation in the Mg^{2+}-dependent ATPase; it could arise because catalytic amounts of proteins, such as actin, or alternative forms of ATPase may allow the reversal of ATP binding, giving rise to the observed rate constants. Both groups are attempting to resolve this discrepancy by designing new experiments.

Cohn: What is the concentration of ATP relative to ADP in all forms in the experiment of Mannherz *et al.*?

Trentham: The ratio of all forms of ATP to all forms of ADP is about 5×10^{-3} at 20 °C and pH 8 in the presence of $MgCl_2$ and KCl.

Cohn: This differs grossly from the value one would find with only catalytic amounts of enzymes.

Trentham: Yes; this ratio also depends on the concentration of phosphate and the pH.

Hess: Are there any temperature studies on the rate constants and dissociation constants?

Taylor: There are some for k_{+2} and K_1 (the equilibrium constant K_1 is measured because the first step is fast). The constant k_{+2} shows a normal dependence on temperature. The rate-limiting step is easy to measure with respect to temperature. The experiments have been done with myosin and S-1; our results closely resemble those of Bagshaw *et al.* (1974) when one allows for the different ionic strengths.

Roseman: Is the covalent linking of phosphoryl enzyme ruled out?

Taylor: It is not absolutely ruled out as long as a phosphorylated state has a

short half life. But, in the steady-state, it is essentially ruled out by other evidence.

Trentham: The work of Boyer *et al.* (1974) with $H_2{}^{18}O$ indicates that water enters the scheme at the step labelled K_3 (see p. 78).

Roseman: We still encounter the obstacle of not knowing whether unidentified intermediary forms exist; K_3 could represent more than one reaction.

Trentham: In practice, it must do so, because the inorganic phosphate molecule in M**·ADP·P_i contains three to four atoms of ^{18}O when ATP is treated with myosin in the presence of $H_2{}^{18}O$, so that the phosphate group must be capable of some rearrangement (Sartorelli *et al.* 1966; Bagshaw & Trentham 1973).

Cohn: Boyer has stated that ^{18}O entered the P_i by reversal of the K_3 step, as it is much more rapid than the following step.

Trentham: I agree, except that the phosphate group must rearrange so that the same ^{18}O atom doesn't come out. This is the principle of microscopic reversibility.

Glynn: Is this kind of approach available for those of us who work with insoluble preparations, for example, with ion pumps in membranes?

Gutfreund: If one can prepare suspensions, one can observe the fluorescence or study the changes in transmission in a double-beam instrument.

Huxley: Can you get sufficient concentrations of bound ATP and other membrane-bound components to measure?

Gutfreund: Yes, especially for the measurement of fluorescence. The technique is enormously sensitive, for example we can use 0.1 μmol l^{-1}. An alternative method for unpleasant mixtures is rapid sampling, by which one can measure the rates of chemical changes.

Huxley: Can one detect bound ATP by starting with a membrane preparation, ADP and P_i?

Trentham: Yes; Boyer's group is currently doing this (Boyer *et al.* 1974).

Kornberg: If you are defeated in your attempt to find bound ATP or to measure K_2 directly, why don't you use analogues like β,γ-methylene-ATP which are not readily hydrolysed?

Trentham: We do; but then we are not looking at ATP.

Gutfreund: Kinetic experiments have been done with various derivatives.

Kornberg: But you would at least have a qualitative indication whether the mythical M*·ATP exists.

Trentham: The evidence for that is available. As we described (p. 77), we used ATP(γS), which is slowly hydrolysed. By that, we specifically mean that the steady-state complex contains uncleaved ATP(γS). The complex is the analogue of M*·ATP.

Gutfreund: During the reaction with ATP, the steady-state complex is $M^{**}\cdot ADP\cdot P_i$ but in the reaction with ATP(γS) the steady-state complex is a $M^{*}\cdot ATP(\gamma S)$ complex.

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Magnetic resonance studies of specificity in binding and catalysis of phosphotransferases

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Abstract Two common characteristics of the active site structures of intermediate complexes formed in kinase reactions have been observed by magnetic resonance techniques. First, in creatine, arginine, adenylate and pyruvate kinases (EC 2.7.3.2, 2.7.3.3, 2.7.4.3 and 2.7.1.40, respectively) water is progressively excluded and the structure at the active site is progressively immobilized as each reactant is successively added to the enzyme, as monitored by electron spin resonance (e.s.r.) and the enhancement of the proton relaxation rate of water (PRR) due to paramagnetic manganese(II) probe. Significant, and often widespread, changes in the protein conformation accompanying successive additions of reaction components are shown with ^{1}H n.m.r. studies of pyruvate kinase. The second characteristic is that, for the ternary enzyme–Mn–nucleotide complexes, two parameters, the e.s.r. spectrum and PRR enhancement values, fall within a range of 10% for all enzymes investigated, with the exception of bovine brain creatine kinase. These similarities suggest a homology in tertiary structure at the active sites of these enzymes.

An unsuspected aspect of substrate and cofactor specificity has been revealed by e.s.r. spectroscopy of the manganese(II) complexes of the transition-state analogue of creatine kinase (E–MnADP–formate–creatine) and of the ternary phosphoenolpyruvate complex. In the former case, replacement of ADP, the normal substrate, by its substrate analogues IDP or 2′-deoxyadenosine diphosphate produced two interconvertible species of the transition-state analogue complexes, observed in the e.s.r. spectra as an isotropic species and a highly anisotropic species. With the normal substrate, only the anisotropic species is observed. Similarly, in the case of the complex pyruvate kinase–Mn–phosphoenolpyruvate, when the normal monovalent activator K^+ is replaced by the inert tetramethylammonium ion, again two interconvertible species rather than the normal one species are observed by e.s.r. spectroscopy. The implications of these phenomena for the relation of specificity to catalytic efficiency are discussed.

Nucleoside triphosphates are the almost universal currency for energy transfer in living organisms. The formation of ATP from ADP and inorganic phosphate is coupled with energy-producing reactions such as oxidation through the

electron transport system and glycolysis. Conversely, the expenditure of the chemical potential energy of the nucleoside triphosphates by conversion into the corresponding diphosphates and inorganic phosphate, or alternatively the monophosphate and inorganic pyrophosphate, is coupled to many energy-requiring processes. Those processes that are characterized by the transfer of chemical energy derived from hydrolysis of the nucleoside triphosphates to other forms of energy, such as mechanical work in muscle contraction and translocation on the ribosome during protein synthesis or ion transport as exemplified by the Na^+,K^+-ATPase, are of necessity complex processes of cellular organelles. However, those processes in which chemical energy is transferred for endergonic chemical reactions, such as biosynthesis of macromolecules and phosphoryl transfer in metabolic regulation, are in general simpler and, consequently, more amenable to detailed analysis at a molecular level.

Of about 100 characterized kinases which catalyse reactions of the type (1)

$$\text{ATP} + \text{X} \rightleftharpoons \text{X–P} + \text{ADP} \tag{1}$$

I shall concentrate particularly on those that control the concentration of ATP; other reactions of nucleoside triphosphates will be alluded to for comparison.

The biosynthetic pathways for all macromolecules, such as proteins, carbohydrates, phospholipids and nucleic acids, share the general reaction (2) with a nucleoside triphosphate (NTP), as exemplified in protein synthesis (reaction 3).

$$\text{NTP} + \text{X} + \text{Y} \rightleftharpoons \text{X–Y} + \text{NMP} + \text{PP}_\text{i} \tag{2}$$

$$\text{ATP} + \text{amino acid} + \text{tRNA} \rightleftharpoons \text{aminoacyl-tRNA} + \text{AMP} + \text{PP}_\text{i} \tag{3}$$

The AMP formed in such reactions must be recycled to form ATP. In the biosynthesis of many small molecules, an analogous reaction (4) produces ADP, as in the synthesis of *N*-formyltetrahydrofolate (reaction 5) with *N*-formyltetrahydrofolate synthetase (EC 6.3.4.3).

$$\text{NTP} + \text{X} + \text{Y} \rightleftharpoons \text{X–Y} + \text{ADP} + \text{P}_\text{i} \tag{4}$$

$$\text{ATP} + \text{formate} + \text{tetrahydrofolate} \rightleftharpoons \textit{N}\text{-formyltetrahydrofolate} + \text{ADP} + \text{P}_\text{i} \tag{5}$$

The AMP formed in the first class of reactions and ADP formed in the second class of reactions may be reconverted into ATP by the reversible reaction $\text{ATP} + \text{AMP} \rightleftharpoons 2\text{ADP}$, catalysed by adenylate kinase (EC 2.7.4.3), a reaction which we have investigated with magnetic resonance techniques (Cohn *et al.* 1971; Price *et al.* 1973, 1975). Although the main pathways for regenerating ATP from ADP are by oxidative phosphorylation and glycolysis, the sole pathway of regenerating ADP from AMP is the adenylate kinase reaction.

Adenylate kinase is ubiquitous and essential for the life of the organism, since without it all biosynthesis ceases. Through its control of the degree of phosphorylation of the nucleotides beyond the monophosphate level, it controls many metabolic processes. It possesses the unique property of catalysing phosphoryl transfer with ADP as donor; it is the only enzyme which can use the energy of the phosphoanhydride bond of ADP for phosphoryl transfer.

Another enzyme which controls the concentration of ATP is creatine kinase (EC 2.7.3.2) which catalyses the reversible reaction ATP + creatine $\rightleftharpoons$ ADP + phosphocreatine. Presumably, arginine kinase (EC 2.7.3.3) is the analogous enzyme in invertebrates. Both enzymes have been examined in considerable detail.

For several years, we have used magnetic resonance techniques to investigate the interaction of substrates with enzymes (Cohn 1970; Cohn & Reuben 1971; Cohn *et al.* 1971). The observations that all enzymic reactions of nucleoside triphosphates require a bivalent metal ion for activity and that the paramagnetic manganese(II) ion can always serve as an activator encouraged the use of magnetic resonance spectroscopic techniques with this paramagnetic probe to investigate properties of the enzyme–metal–substrate complexes. With such paramagnetic probes we can study (1) binding constants and the number of binding sites for metal ions and substrates, (2) the environment of the metal ion, (3) the structures and configurations at the active site of various enzyme–metal–substrate complexes and the disposition of the two substrates with respect to each other on the surface of the enzyme, (4) the mobility at the active site and ligand exchange rates of these complexes (i.e., the dynamic properties of the active complexes) and (5) the role of the metal ion in enzyme catalysis. In this paper, I shall emphasize some common features which have been established thus among these enzymes in regard to three aspects of these systems: (1) the effects of substrate binding on the structure of the complex at the active site, (2) the properties of nucleotide-binding sites and (3) substrate specificity and its relation to catalysis.

One of the most striking common features of kinase reactions is the observation that the binding of each additional ligand to the enzyme progressively changes the structure at the active site so that the substrates become more immobilized and the active site becomes less accessible to water. Table 1 lists the values of the enhancement factors, ε, of the relaxation rate of water protons (PRR) (Mildvan & Cohn 1970) for various Mn(II) complexes with several enzymes. A similar pattern emerges for creatine kinase (Reed & Cohn 1972), arginine kinase (Buttlaire & Cohn 1974*a*) and adenylate kinase (Price *et al.* 1973). There is a weak binding of Mn(II) in the absence of substrates with a low enhancement factor. The highest value of the enhancement factor (ε_t) is

TABLE 1

Relaxation rates of water protons, expressed as enhancement factors, ε, of enzyme–Mn–substrate complexes of several kinases

Kinase	*Binary complex*		*Ternary complex*		*Quaternary complex*		*Transition-state analogue*	
	Complex	ε_b	*Complex*	ε_t	*Complex*	ε_q	*Complex*	ε_{tr}
Creatine								
from rabbit muscle[a]	E–Mn	6.5	(I) E–ATP–Mn	10–15			(II)–creatine–HCO_3^-	6.3
			(II) E–ADP–Mn	20.5	(II)–creatine	12.5	(II)–creatine–NO_3^-	5.1
from bovine brain[b]	E–Mn	5.4	(II) E–ADP–Mn	9.1	(II)–creatine	6.0	(II)–creatine–NO_3^-	5.0
Arginine								
from *Homarus americanus*[c]	E–Mn	9.6	(I) E–ATP–Mn	16.7				
			(II) E–ADP–Mn	18.9	(II)–L-Arg	9.4	(II)–L-Arg–NO_3^-	4.6
Adenylate								
from porcine muscle[d]	E–Mn	4.0	(I) E–ATP–Mn	14.8	E–Ap_5A–Mn[f]	5.0		
Pyruvate								
from rabbit muscle[e]	E–Mn	25	E–Mn–pyruvate	5.10				
			E–Mn–phospho-enolpyruvate	2.3				

[a] Reed & Cohn (1972). [b] Markham & Reed, unpublished experiments. [c] Buttlaire & Cohn (1974*a*). [d] Price *et al.* (1973). [e] Mildvan & Cohn (1966); James *et al.* (1973). [f] Ap_5A is diadenosine pentaphosphate.

observed for the ternary ADP complex which is reduced (ε_q) on addition of the second substrate to form the abortive quaternary complex in creatine and arginine kinase. A similar reduction is observed when the multisubstrate inhibitor diadenosine pentaphosphate is added to adenylate kinase. Little effect on the enhancement of the binary E–Mn complex, ε_b, is observed when the non-nucleotide substrate is added to E–Mn to form a ternary complex with, for example, creatine or phosphocreatine. Addition of the second substrate to the ternary complex E–Mn–ADP obviously affects the micro-environment of the first substrate (ADP) which is liganded to Mn(II). The addition of nitrate or formate to the abortive quaternary complex of creatine kinase and of nitrate to the analogous arginine kinase complex further lowers the enhancement factor. These anions are presumably analogues of the phosphoryl group in the transition state, as first noted by Milner-White & Watts (1971). So, we conclude that the structures of the active sites in the intermediate complexes in a sequence of steps in an enzymic reaction may differ considerably from each other and from the structure in the transition state.

Pyruvate kinase (EC 2. 7. 1. 40) differs in detail from the other kinases (see Table 1) since it forms a stronger binary complex with Mn(II) with a large enhancement factor ($\varepsilon_b \approx 25$) (Reuben & Cohn 1970) which is strongly affected by binding of pyruvate or phosphoenolpyruvate (Mildvan & Cohn 1966; James *et al.* 1973). It has been proposed that this enzyme is mechanistically homologous to other enzymes with phosphoenolpyruvate as phosphoryl donor (Miller *et al.* 1968; Rose 1972). In the cell, it does not function as a typical kinase since the reaction from the ATP direction is highly unfavourable energetically. In glycolysis, the phosphoryl group is transferred with phosphoenolpyruvate as the donor, and gluconeogenesis does not occur by reversal with ATP as donor. Nevertheless, the pattern of decreasing PRR enhancement factors as successive subsites of the enzyme are occupied holds for this system as well since ε(E–Mn) $>$ ε(E–Mn–pyruvate) $>$ ε(E–Mn–phosphoenolpyruvate).

The interpretation of the paramagnetic contribution to the relaxation rate of water protons in terms of molecular parameters of enzyme complexes has been discussed in general (Mildvan & Cohn 1970) and in detail (Reuben & Cohn 1970). Among the parameters that can be estimated in some cases is the number (n) of aqueous or equivalent protons in the first coordination sphere of Mn(II) which are exchangeable with solvent water. Table 2 lists the values of n for those complexes for which it could be estimated; note that n decreases as the number of protein ligands increases, for example, from 2 for the ternary nucleotide complexes of arginine kinase through 1 for the abortive quaternary

TABLE 2

Apparent number (n) of water ligands coordinated to Mn (II) in enzyme–substrate complexes

Enzyme	*Complex*	n
Arginine kinase	E–MnADP	2
	E–MnATP	2
	E–MnADP–L-arginine	1
	E–MnADP–L-arginine–NO_3^-	0.3–0.4
Creatine kinase	E–MnADP–creatine–NO_3^-	0.3–0.4
Pyruvate kinase	E–Mn	3
	E–Mn–phosphoenolpyruvate	0.2–0.5
N-Formyltetrahydrofolate synthetase[a]	E–MnATP	2 ± 1
	E–MnADP–tetrahydrofolate	0.35
	E–MnATP–tetrahydrofolate	0.35
	E–MnADP–tetrahydrofolate–formate	0.03

[a] Buttlaire *et al.* (1975).

complex with ADP and L-arginine to 0.35 for the transition-state analogue with nitrate. The same pattern holds for all the enzymes. The *N*-formyltetrahydrofolate system is particularly striking in that, at the active site, Mn(II) is so completely shielded from the solvent that it has practically no effect on the PRR. Values of $n < 1$ can be explained in two ways: (1) residual water molecules in the first coordination sphere exchange too slowly with solvent water to affect the PRR or (2) all water molecules in the inner coordination sphere have been replaced by protein ligands and the small effects observed are due to outer sphere effects or to protein protons exchangeable with water in the Mn(II) environment. At present, there is no way of distinguishing between the two possibilities but either explanation is associated with an active site-structure which is less flexible owing to either protein ligands to the metal ion or another structural change which makes the metal ion inaccessible to the solvent.

The changes in e.s.r. spectra of Mn(II) in various complexes complement the relaxation data well. Increasing asymmetry and immobilization are observed as substrates are added to the system in creatine kinase (Reed & Cohn 1972), arginine kinase (Buttlaire & Cohn 1974*b*), adenylate kinase (Price *et al.* 1973) and pyruvate kinase (Reed & Cohn 1973). The highly anisotropic e.s.r. spectrum of the transition-state analogue complexes for creatine kinase, that is E–MnADP–creatine–NO_3^- or –HCO_3^-, relative to the ternary E–Mn–ADP

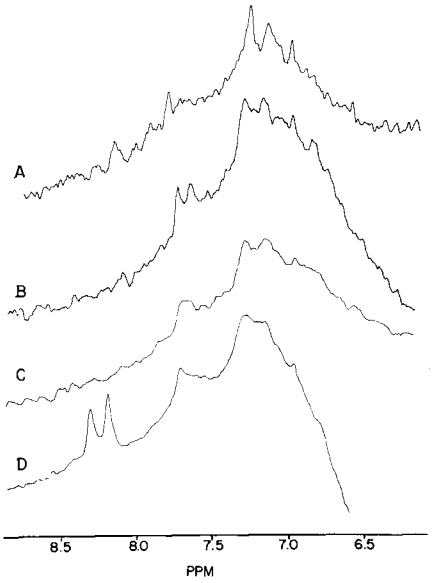

FIG. 1. Effect of various ligands on the region downfield from water of the ^1H n.m.r. spectrum of pyruvate kinase from rabbit muscle. The following components were added to the enzyme solution, pH~7.5 (about 100 mg pyruvate kinase/ml): (A), 5mM-tetramethylammonium phosphate, 20mM-tetramethylammonium chloride; (B), 100mM-KCl, 5mM-potassium phosphate; (C), 100mM-KCl, 7mM-$MgCl_2$, 10mM-potassium phosphate; (D), 100mM-KCl, 7mM-$MgCl_2$, 2mM-phosphoenolpyruvate.

complex supports the conclusion that additional ligands on the Mn(II) are donated from the protein to immobilize the substrate in the transition state.

The effects of ligand binding on the conformation of the protein—the progressive changes with successive addition of each ligand—may be directly observed in the ^1H n.m.r. spectra of the protein in various complexes. Fig. 1 shows such spectra of pyruvate kinase from rabbit muscle in the region of the magnetic field below the H_2O resonance which includes the signals from the histidine and aromatic residues, tryptophan, phenylalanine and tyrosine. Pyruvate kinase requires a monovalent cation as well as a bivalent cation for activity; potassium ions appear to be the natural activator and tetramethylammonium ions are completely inert. In Fig. 1A, the enzyme solution contains only tetramethylammonium phosphate but when KCl is present (Fig. 1B) the K^+ introduces significant changes which become far more profound as $MgCl_2$ (Fig. 1C) and phosphoenolpyruvate (D) are added. Although these changes cannot yet be assigned to specific amino acid residues, the number of peaks

which are perturbed and, consequently, the number of residues whose environment must be affected by each additional ligand is impressive.

Of all the kinases, adenylate kinase is the most amenable to investigation by high resolution n.m.r. spectroscopy because it is the smallest known, with a molecular weight of about 21 000 daltons. The enzyme from rabbit muscle, porcine muscle and carp muscle contain three, two and one histidine, respectively, and none contains tryptophan. Consequently, the ^1H n.m.r. spectra of the aromatic region are particularly well resolved. The enzyme from porcine muscle is also the only kinase for which both the X-ray crystal structure at 0.35 nm (Schultz *et al.* 1974) and the amino acid sequence (Heil *et al.* 1974) have been determined. The histidine which is conserved (His-36) has a normal titration curve ($pK \sim 6.2$) but His-189 is not titrable in the usual range. The binding of the substrate GTP changes the pK of His-36 about 1 pH unit. Strong evidence that His-36 is at, or close to, the active site is provided by the finding that MnATP affects the C-2 proton resonance (i.e. its relaxation rate) at lower concentrations than for any other observable proton. Manganese(II) in the absence of ATP does not induce this effect. Further work is in progress to delineate the active site in more detail by n.m.r. spectroscopy.

Rossman *et al.* (1974) suggested that nucleotide-binding sites on all proteins have common structures. Table 3 lists the enhancement factors, ε_t, and disso-

TABLE 3

Comparison of enhancement factors for the relaxation rate of water protons (PRR), ε, and dissociation constants, K_d, for the various ternary complexes, E–Mn(II)–nucleotide

Enzyme	*MnADP complex*		*MnATP complex*	
	ε_t	K_d/μmol l^{-1}	ε_t	K_d/μmol l^{-1}
Creatine kinase				
from rabbit muscle	20.5	64	10–15	
from beef brain	9.6	8		
Arginine kinase				
from *Homarus americanus*	18.9	7	16.7	79
from *Panulirus longpipes*[a]	18.0	30		
Adenylate kinase				
from rabbit muscle			14.8	44
from porcine muscle			14.0	30
Phosphoglycerate kinase				
from yeast[b]	21.0	5		
N-Formyltetrahydrofolate synthetase[c]	19.8	175	12.5	64

[a] W. J. O'Sullivan, personal communication. [b] Chapman *et al.* (1974). [c] Buttlaire *et al.* (1975).

ciation constants, K_d, of the manganese(II) nucleotide for eight ternary complexes. With the exception of that for creatine kinase from beef brain, the ε_t values for the MnADP complexes fall in the narrow range 18–21 and the values for the ATP complexes cover a somewhat larger range of 12.5–16.7. Since the K_d values of the ADP complexes range from 5 to 175 μmol/l, it appears that ε_t, which is a function primarily of the geometry as prescribed by the tertiary structure at the active site, is similar for all the enzymes but K_d, which is influenced predominantly by the primary structure, varies 35-fold.

Indirect evidence about the homology of the detailed structure of the nucleotide-binding site may be derived from the specificity of the nucleotide substrates. A wide spectrum of specificities exists among the kinases. For instance, the triphosphate subsite of adenylate kinase is rather unspecific since all the usual bases are substrates; in contrast, the monophosphate subsite is highly specific for adenine. For creatine kinase, when IDP (which has about 20% the activity of ADP) forms a transition-state analogue complex, E–MnIDP–creatine–formate (Reed & McLaughlin 1973), the e.s.r. spectrum revealed two interconvertible species, an isotropic form and a highly anisotropic form. The latter is similar to the single anisotropic species observed in the analogous MnADP complex.

The same phenomenon has been encountered with dADP in its E–Mn–dADP–creatine–formate complex, which was more suitable for quantitative investigation than the IDP system because the binding of dADP to the enzyme is much stronger than that of IDP. A study of the temperature dependence of the equilibrium between the two forms of the dADP complex showed that, at low temperature, the equilibrium was shifted almost entirely to the anisotropic form, that is, to the same type of species that is observed at all temperatures with ADP.

If the conversion of the isotropic, supposedly inactive, species is a rate-determining step in the reaction of dADP, then it should be possible to observe the effect of the interconversion on the kinetics of the reaction. From Fig. 2, the rates of the reaction with phosphocreatine of ADP and dADP may be compared as a function of temperature. Similarly, the amount of the anisotropic species for both the ADP and dADP transition-state analogue complexes have been measured from the e.s.r. spectra as a function of temperature. For both parameters, the ADP and dADP values overlap at low temperatures and deviate at higher temperatures. The correspondence between the temperature dependence of the reaction velocity and of the amount of anisotropic species is gratifying.

A somewhat analogous phenomenon of specificity has been observed for pyruvate kinase. If the monovalent cation activator potassium is replaced by

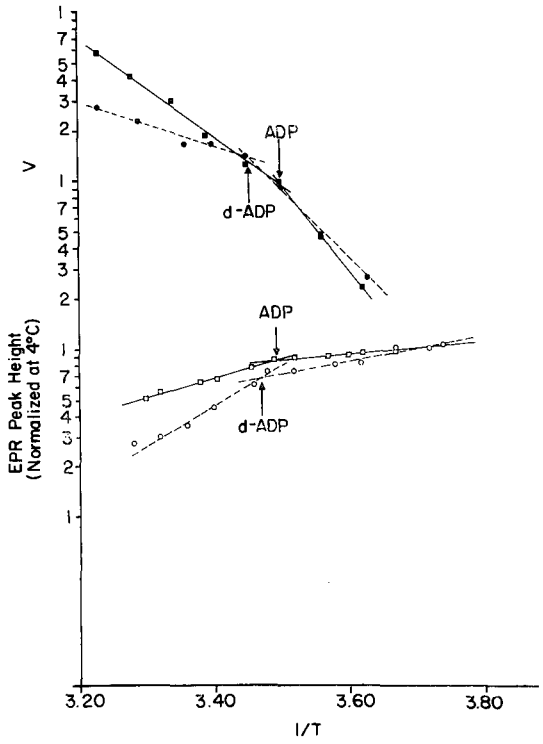

FIG. 2. Comparison of the temperature dependence of the rates (V) of the Mn(II)-activated reverse creatine kinase reaction (■—■ ADP; ●--● dADP) with the temperature dependence of the Mn(II) peak amplitudes of the e.s.r. spectra of the transition-state analogue E–MnADP–creatine–formate (□—□ ADP; ○--○ dADP). The peak heights in the e.s.r. spectrum are those due to the anisotropic portion of the spectrum at low magnetic field and the value for the peaks of ADP and dADP complex were normalized at 4 °C. The arrows indicate the temperature of the break point in the biphasic curves.

the inert tetramethylammonium ion $(CH_3)_4N^+$, in the E–Mn–phosphoenolpyruvate complex, two interconvertible species appear in e.s.r. spectra (Reed & Cohn 1972). Again, one species has a highly anisotropic spectrum which corresponds closely to the single form of the K^+-complex. In this case, the equilibrium is shifted towards the anisotropic form when the temperature is raised. Reed & I (1972) were able to determine the equilibrium constant quantitatively since the methylene analogue of phosphoenolpyruvate displayed only the isotropic spectrum in the presence of either K^+ or $(CH_3)_4N^+$, thus making it possible to follow the temperature dependence of the e.s.r. spectrum of isotropic species alone. Other ions, such as Na^+, which are intermediate

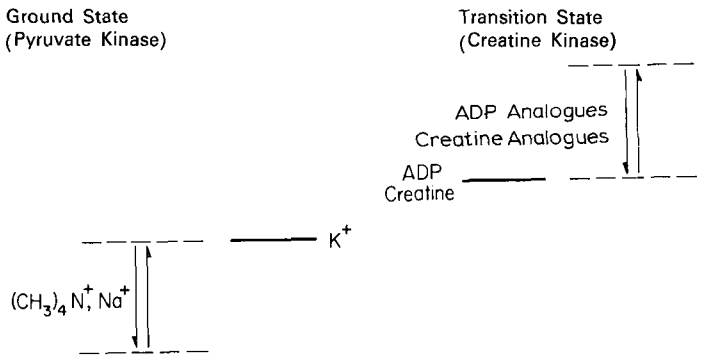

FIG. 3. Energy diagram comparing normal substrates and analogues in the ground state and transition state.

activators, also produced an intermediate amount of the anisotropic species. I should point out that the existence of the anisotropic species is a necessary but not sufficient condition for enzymic reactivity. The $(CH_3)_4N^+$ complex, about 80% of which exists in the anisotropic form at 25 °C, is nevertheless completely inactive. Unlike the creatine kinase system, this equilibrium was not reflected in the temperature dependence of the kinetics of pyruvate kinase determined for several monovalent cation activators.

In the E–Mn–phosphoenolpyruvate complex, which is in the ground state, the temperature must be raised to shift the equilibrium from the 'inactive' to the 'active' form but in the transition-state analogue of creatine kinase the temperature must be lowered to shift the substrate analogue complex to the active form. From these findings we may infer that enzymes are designed to catalyse their normal substrates optimally. This concept is schematically diagrammed in Fig. 3; the energy of the ground state is higher for the normal activator K^+ than for the tetramethylammonium ion complex for the temperature must be *raised* to form the active species. In the transition state (represented by the creatine kinase transition-state analogues), the analogue with the normal substrate, ADP, is lowest in energy for the temperature must be *lowered* to form the active species. Thus, with the normal reaction components, the energy difference between the ground state and the transition state is minimal, thereby maximizing the catalysis.

At the present time, we have no definite information about the structural differences between the 'inactive' species characterized by isotropic Mn(II) e.s.r. spectra and the 'inactive' species characterized by highly anisotropic e.s.r. spectra. We can speculate that in the 'inactive' species the Mn(II) is directly liganded to the phosphoryl group to be transferred but, in the 'active' species,

the Mn(II) is not so liganded. The basis of this speculation is that the structure of the ternary complex of pyruvate kinase with Mn(II) and an inhibitor, the methylene analogue of phosphoenolpyruvate, exists only in the inactive form in the presence of K^+ or $(CH_3)_4N^+$ and the structure of this has been shown by n.m.r. spectroscopy to be consistent with Mn(II) binding directly to the phosphoryl group (James & Cohn 1974). However, in the normal complexes of creatine kinase, E–Mn–ADP–creatine and E–MnATP–creatine, the n.m.r. data are consistent with Mn(II) being liganded to the α- and β-phosphates but not the γ-phosphate of ATP—in other words, not to the phosphoryl group to be transferred (Cohn *et al.* 1971).

In summary, magnetic resonance studies have revealed several common features of the structures induced at the active site of kinase by the successive binding of substrates and activators as well as common structural characteristics of the nucleotide-binding site of these enzymes. In addition, because of the rapid time scale of e.s.r. spectroscopy, two hitherto unsuspected species that interconvert slowly on the e.s.r. time scale are discernible in some substrate-analogue complexes. The temperature dependence of these interconversions has led to the suggestion of a new aspect of the relation of substrate specificity to catalysis.

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Discussion

Gutfreund: What sort of distances around the active site can one study by n.m.r. spectroscopy?

Cohn: In n.m.r. we can get information about the effect of manganese on the protein protons over about 1.5–2.0 nm.

Gutfreund: Although you said that tighter binding was the evidence for the transition-state analogue, surely, if there were any obligatory order of binding, your argument would not hold?

Cohn: The order of binding is not obligatory in these enzymes. The binding constants of some creatine analogues, for example, differ by two orders of magnitude in the presence and absence of nitrate in the enzyme–MnADP–creatine analogue complexes. Another factor which supports the transition-state analogue interpretation is the specificity of the ion. Geometrical considerations demand a planar ion. Also, the ion should be lipid-soluble. Nitrite, nitrate and thiocyanate ions satisfy these conditions; formate participates in complex formation, but not so well. These ions are also bound tightly themselves: the binding constant for nitrate is in the micromolar range. Milner-White & Watts (1971) showed that these anions had a kinetic effect. The addition of low concentrations of nitrate to a reaction that is proceeding in the direction of forming phosphocreatine stops the reaction dead as soon as a little ADP has been produced, by forming the quarternary complex with nitrate. Addition of a low concentration of ADP from the outset prevents the reaction from being initiated. Thus, nitrate is a potent inhibitor in the presence of ADP.

Hess: How far can you extrapolate information from the manganese enzyme to the magnesium enzyme?

Cohn: That is difficult to answer because I have no way of monitoring the magnesium directly, except by n.m.r. spectroscopy. We can study the n.m.r. spectra of magnesium complexes, but not those of manganese, because high concentrations of manganese obliterate the whole spectrum. As magnesium ions have no effect, we add magnesium ATP and then small amounts of manganese, so that we are really looking at the effect of manganese on the magnesium complex. Unfortunately, with these methods we cannot use comparable amounts of magnesium and manganese.

Generally in kinase reactions, at low concentrations, manganese is a better activator than magnesium but at high concentrations it is a potent inhibitor. Manganese may bind to the sites on protein to which magnesium does not. Also, magnesium tends in general to accept nitrogen ligands in contrast to magnesium which does not. Magnesium is rarely inhibitory and then only at considerably higher concentrations than those at which manganese inhibits. The proteins seem to contain weak binding sites to which manganese will bind and thus inactivate the enzyme.

A qualitative difference in the kinetics is observed on the addition of manganese and magnesium to pyruvate kinase but not to other enzymes. Most likely, the function of manganese and magnesium in pyruvate kinase differs

considerably from that in most other kinases: the metal ion binds directly to the enzyme—it is not directly attached to ATP (A. S. Mildvan, unpublished findings, 1973). I maintain that phosphoenolpyruvate is not a ligand of manganese in the ternary enzyme complex, but I still have to prove that. In the other kinases, without a doubt, manganese is liganded to ATP. The pyruvate kinase pattern (i.e. the formation of a binary E–M complex) follows that of other enzymes with phosphoenolpyruvate and pyruvate as substrates. These are metalloenzymes, in which the enzyme binds to the metal directly in a binary complex in contrast to kinases. Pyruvate kinase is such an exceptional kinase, that I suspect that mechanistically it should not be classified as a kinase at all!

Taylor: In view of the hypothesis that nucleotide-binding sites have common structures (Rossman *et al.* 1974), how much is known about the site of the thiol group? I know you have located these in CP kinase and they have been located in the crystal structure of adenylate kinase (EC 2.7.4.3).

Cohn: Price, Schirmer & I (1975) have extensively investigated the environment of the thiol groups in adenylate kinase using a fluorogenic probe, 7-chloro-4-nitrobenzo-2,1,3-oxadiazole (NBD chloride). The enzyme from porcine muscle, for which the X-ray crystallography has been done, contains two thiols. Fortunately for us, the carp enzyme contains only one histidine and one thiol group. The residues that are conserved in the carp enzyme are histidine 36 and cysteine 25. Cysteine 25 reacts about 40 times faster with the fluorogenic reagent than does the cysteine 187 which lies on the outside of the molecule and reacts at the same rate as glutathione does. Cysteine 25 reacts at about the same rate as does the cysteine at the active site in glyceraldehyde-3-phosphate dehydrogenase.

Another unusual feature is that adenylate kinase is the only enzyme modified by reaction with NBD chloride in which the NBD group is transferred from the sulphur atom of the thiol to a nitrogen atom. Such a transfer occurs in several model compounds such as cysteine and homocysteine. The transfer on the enzyme implies a proximate amino group, which X-ray crystallography confirms. Lysine 31 is a close neighbour of cysteine 187 (about 0.4 nm away) on the exterior of the molecule.

Diadenosine pentaphosphate, Ap_5A, is an excellent inhibitor of adenylate kinase (Lienhard & Secemski 1973). It also reduces the rate of reaction of NBD chloride with the thiol group in cysteine 25 by a factor of 300. The tetraphosphate, Ap_4A, which one might expect to be a good inhibitor, is not, but Ap_6A is, like Ap_5A, excellent. Looking at the model built from crystallographic data, I predict that Ap_7A will not inhibit as it is too large to fit into the active site.

Taylor: Do you consider that these thiols are necessary for catalysis?

Cohn: They are not essential. When both thiols are blocked by some reagents, as much as 40% activity remains (Kress *et al.* 1966). We found that blocking cysteine 187 with NBD chloride leaves the activity unaltered, whereas blocking cysteine 25 with the same reagent eliminates all activity. In the kinase reactions in general, not one single thiol has been shown to be essential for catalytic activity. With creatine kinase whose activity has been eliminated by carboxymethylation of one thiol group, Smith & Kenyon (1974) completely blocked one thiol by *S*-methylation but found that the enzyme still had 20% activity. Quiocho & Thompson (1973) blocked another thiol yet found 100% activity remained. On modification of the thiols in adenylate kinase, the structure changes; the protein becomes unstable and slowly precipitates. If we block the thiols in creatine kinase, the ability of the substrate, creatine, to cause the normal conformational change when it is bound in the enzyme–MnADP complex is lost, as evidenced by the e.s.r. spectra of Mn(II). Similarly, the proton relaxation rate of water changes in the same complex with native enzyme but does not change with the modified enzyme. Thus, although the modified enzyme still binds creatine (as shown by spin label experiments), creatine can no longer induce the essential conformational change that occurs in the native enzyme. I predict that in no kinase reaction will the thiol group participate directly in catalysis.

Glynn: Do the non-phosphorylating ATP analogues which are not hydrolysed (such as β,γ-methylene-ATP or the corresponding imido analogue) interact in a similar way?

Cohn: Unfortunately, neither the β,γ-methylene compound nor the nitrogen analogue will bind to creatine kinase. The α,β-methylene analogue, however, is an excellent substrate, behaving similarly to ATP.

Phillips: What is the concentration of protein in the n.m.r. spectroscopy experiments?

Cohn: We have been using 1mM-protein (that is about 20 mg/ml). We can prepare 4–5 mmol/l solutions of, for instance, arginine kinase. This enzyme is a monomer with a molecular weight of about 38 000 in contrast to creatine kinase which is dimeric with a molecular weight of about 80 000.

Phillips: In typical protein crystals, depending on the amount of liquid present, the protein concentration ranges between 1 and 50 mmol/l. So, the coincidence of the results of crystal studies with those of solution studies does not surprise me. What is the protein concentration *in vivo*?

Cohn: The concentration of adenylate kinase in muscle is about 300 mg/kg but in view of its localization, the concentration is of the order of 0.1 mmol/l.

Hess: I should add that the cellular concentration of the glycolytic enzyme

in yeast is, with some variation, around 0.1 mmol/l. Pyruvate kinase represents about 8% of the total soluble protein in yeast. All glycolytic enzymes in yeast make up about 65% of total soluble protein in this species. Do you see any response on adding monovalent ions?

Cohn: Yes. The protein structure changes when we add potassium ions, but we have not interpreted these changes yet. In contrast, as I explained (pp. 95, 96), when tetramethylammonium ions (which, unlike potassium, neither activate nor bind to the enzyme) are added to the phosphoenolpyruvate enzyme complex, we see two interconvertible complexes in the e.s.r. spectra. There is no question that the monovalent ion has a profound effect on the structure of the complex that contains both the pyruvate and the phosphate groups, i.e. phosphoenolpyruvate, but not pyruvate alone.

Hess: Is potassium definitely necessary for catalysis?

Cohn: Potassium is essential for pyruvate kinase activity. Tetramethylammonium ions abolish the activity.

Kornberg: Potassium is also essential for the FDP-stimulated pyruvate kinase of *E. coli*.

Lipmann: You said (p. 101) that you thought that pyruvate kinase should not be classified as a real kinase. Can you 'reverse' the activity to obtain enolphosphate? There must be enolpyruvate present; the keto form should not be able to accept phosphate from ATP.

Cohn: I am sure that it goes through the enol form. Rose (1960) has shown that pyruvate kinase catalyses keto–enol isomerization, for which all the cofactors must be present: Mg^{2+} (Mn will activate, too), K^+ and also the phosphate site must be occupied. Methyl phosphate and inorganic phosphate will serve. The first step in the reaction from pyruvate is the conversion of the keto into the enol form. Glycolate resembles the enol form and Kayne (1974) has shown that glycolate will serve as substrate for pyruvate kinase. He successfully made the reaction (1) go in the reverse direction at a reasonable rate.

$$\mathrm{ATP} + \mathrm{HO{\cdot}CH_2{\cdot}CO_2^-} \xrightarrow{\text{pyruvate kinase}} \mathrm{P{\cdot}O{\cdot}CH_2{\cdot}CO_2^-} + \mathrm{ADP} \qquad (1)$$

I was being facetious in suggesting that pyruvate kinase is not a true kinase, because it does transfer a phosphoryl group from ATP, but mechanistically it does not behave like most other kinases. For example, nucleotides (ATP or ADP) have no effect on the e.s.r. spectrum of the manganese enzyme. A dramatic effect is observed with phosphoenolpyruvate and with pyruvate, but with the other kinases the nucleotides are essential in their interaction with the metal ion and enzyme before any effect of the second substrate is seen.

Hess: Aren't MgATP and MgADP the two substrates for pyruvate kinase?

Cohn: One needs a magnesium enzyme for binding of ATP. It has not yet been established whether ATP is bound in the ternary complex with Mg as the bridge atom. Mildvan's results with Mn, demonstrating that ATP is not directly bound to Mn in the pyruvate kinase complex, suggest that Mg does not bind both enzyme and ATP in a bridge complex.

Lipmann: Is there much glycolate phosphate as well as ADP formed?

Cohn: Yes. The equilibrium lies far over towards phosphoglycolate.

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Energetic aspects of transport of ADP and ATP through the mitochondrial membrane

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Abstract Evidence from various sources demonstrates that the release of ATP in exchange for the entry of ADP across the mitochondrial membrane is an active process requiring energy. The necessary energy may be derived from the same source of energy as that used for oxidative phosphorylation. The following results will be discussed:—(1) The exchange is asymmetric with respect to the specificity of ADP and ATP in 'energized' mitochondria. From the outside ADP is much preferred to ATP, but from the inside both exchange with equal specificity. This asymmetry is abolished by de-energization of the membrane. (2) The ADP–ATP exchange is about 50% electrogenic: about half the ATP released against ADP is protonated. The excess of negatively charged ATP might prevent ADP from entering mitochondria against a membrane potential. (3) The ratio of ATP to ADP across the inner mitochondrial membrane is higher outside than inside only in the energized state. Variation of the ATP/ADP ratio maintains this difference. (4) The ADP/ATP ratio apparently varies with changes in the membrane potential as measured by Rb^+-distribution. The correlation factor between ΔE changes and this ratio is 0.5, in agreement with predictions from proton-stoichiometry measurements. The ΔpH does not significantly change the distribution ratio. (5) By following the P/O ratio, one can show that energy derived from expelling ATP against the ADP/ATP gradient lowers the amount of ATP synthesis. The P/O ratio is lowered as the imbalance of ATP to ADP increases. (6) The energy difference of the phosphorylation potential of ATP is calculated by various methods to be about 8–12 kJ, depending on the conditions. This free energy is the result of the 'active' transport which corresponds to the release of ATP outside the mitochondria. (7) *In vivo* studies on the distribution of ADP and ATP inside and outside the mitochondria in liver show a corresponding ratio difference of about 15 as predicted from the *in vitro* studies.

The two different energetic problems of the transport of ADP and ATP through the mitochondrial membranes which are discussed—(*a*) the energy transfer for the active transport of ADP and ATP and (*b*) the activation energy as a key to the carrier mechanism—are somewhat related, since the mechanism of the

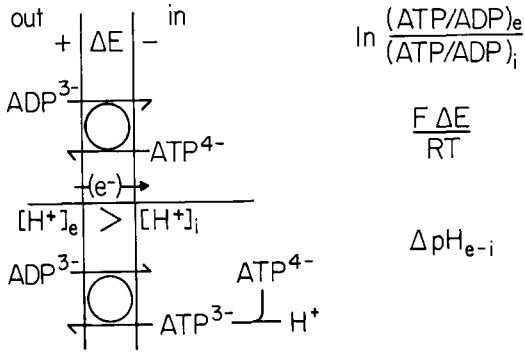


FIG. 1. Energy sources for the gradient of ATP/ADP across the membrane: the ADP/ATP exchange is either an active electrogenic transport driven by membrane potential or an electroneutral transport driven by membrane $\triangle$pH (or both). The figure illustrates the simplest case.

energy transfer for the active transport may be closely linked to the mechanism of carrier catalysis.

Basically, the transport of ADP and ATP through the mitochondrial membrane can function at maximum rate with no energy requirement. The carrier remains a catalyst of transport into which, in certain conditions, energy can be invested. This situation contrasts with some other active transport systems which necessarily depend on the presence of an energy donor such as ATP, for example, in the active Na^+- or Ca^{2+}-pump. However, metabolite transport systems, such as those for glucose, can alternatively operate with or without energy.

ACTIVE TRANSPORT OF ATP AND ADP

The fact that the transport of ATP and ADP through the mitochondrial membrane is energy-linked is shown by the large difference between the ratios of ATP to ADP that can be maintained across the mitochondrial membrane (Fig. 1) (Klingenberg & Pfaff 1966; Heldt *et al.* 1972; Klingenberg 1972). Surprisingly, the ATP/ADP ratio is, in general, higher outside than inside the mitochondria although ATP is first generated inside. This difference in the ratio is abolished as soon as the energy supply from respiration is abolished, for example, with uncouplers.

The mechanism of active transport can be understood mainly on the basis of the different charges of the anions ADP and ATP. Thus, the heteroexchange, ADP–ATP (in contrast to the homoexchange, ADP–ADP or ATP–ATP),

causes a net movement of charge across the membrane: in the exchange of ADP for ATP, one negative charge per ADP–ATP pair can be moved outside (see Fig. 1). Since a potential, ΔE, is generally assumed to exist across the mitochondrial membrane, this may be the source of energy in the electrogenic exchange of ADP for ATP that drives the negative charge towards the positively charged outer side of the membrane. In this way, the external ratio ATP/ADP increases over the internal ratio and at equilibrium this ratio is simply related to the membrane potential.

Fig. 1 also illustrates that in an electroneutral exchange of ADP for ATP the pH dependence across the membrane influences the distribution gradient. Since, normally, it is assumed that the H^+ concentration outside, $[H^+]_e$, is higher than the internal concentration, $[H^+]_i$, the ATP/ADP ratio should be higher inside than outside, contrary to what is observed. Therefore, the main factor sustaining the ATP/ADP ratio is probably the membrane potential.

However, evidence points to both electrogenic and electroneutral elements in the transport of ADP and ATP; so, the total ATP/ADP ratio will be controlled by the opposing forces of the membrane potential, ΔE, and the difference in hydrogen ion concentration, ΔpH. Since the contribution of ΔE is generally greater than that of ΔpH, a higher external ratio of ATP to ADP should be maintained, even if the electrogenic and electroneutral parts of the exchange are equal.

The dependence of $(ATP/ADP)_e/(ATP/ADP)_i$ on the membrane potential has been experimentally verified (see Fig. 2). The membrane potential ΔE, was calculated from the distribution across the mitochondrial membrane of K^+, which can be electrogenically transported with the help of valinomycin. The K^+ distribution was marked by Rb^+ (Padan & Rottenburg 1973). As the external concentration of K^+ is increased, the membrane potential decreases in parallel with the double ratio. From the slope in Fig. 2, the electrogenic contribution, α, is estimated to be 0.6. (In other experiments, α is sometimes as low as 0.3). However, ΔE is the major source of energy for maintaining the gradient of the ATP/ADP ratios.

Since it can be assumed that the energy potential at the membrane is reflected by the internal ratio of ATP to ADP, the external/internal ratio should be related to the internal ATP potential Π (equation 1) (Klingenberg & Pfaff 1966; Klingenberg 1972) (where Π represents the phosphorylation potential

$$RT\ln(ATP/ADP)_e/(ATP/ADP)_i = b\Pi_i$$

$$= b[\Pi_o + RT\ln(ATP/ADP)_i + RT\ln P_i] \quad (1)$$

and b the correlation factor), since the external ratio increases to a greater

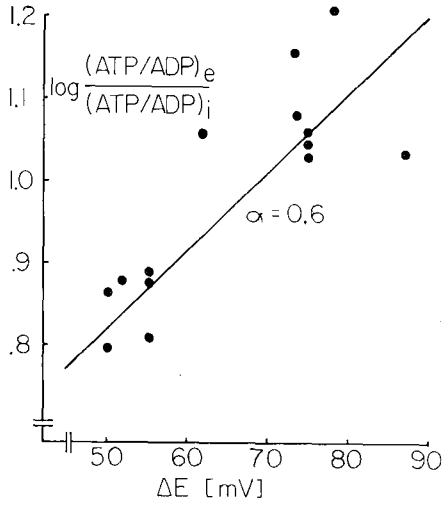


FIG. 2. The dependence of the ratio of the external ratio of ATP/ADP to the internal ratio in rat liver mitochondria on the membrane potential ($\triangle E$; measured by following the distribution of rubidium ions in the presence of valinomycin). For equilibrium measurements [^{14}C]ATP was added. The mitochondria were separated by centrifugal filtration through a layer of silicone. The membrane potential was varied by changing the concentration of K^+ from 2- to 20-mM-KCl (E. M. Klingenberg, B. Schmiedt & H. Rottenberg, unpublished results).

extent than the internal ratio. In the electroneutral ADP–ATP exhange H^+ should be released to the outside with the ATP (see Fig. 1). The stoichiometry of this release should yield information about the extent of participation of this transport. The results shown in Fig. 3 demonstrate that about 0.2–0.4 H^+ are released for every ATP exchanged for ADP. The higher values are more representative of the true ratio since back-leakage of H^+ due to several factors, in particular an influx of P_i, tends to decrease this ratio. Accordingly, when the transport of P_i is inhibited by *N*-ethylmaleimide, the highest ratio is obtained. This stoichiometry is also in agreement with the data from the membrane potential relation (Fig. 2) which show about 50% of the exchange as electrogenic. Possibly, this proportion varies, depending on the other parameters (see later).

Another aspect is the question, to what extent does active transport of ATP compete with ATP synthesis by oxidative phosphorylation for the energy derived from the respiratory chain? This competition is illustrated in Fig. 4; the total energy generated by the respiratory chain is divided between ATP synthesis, transport and 'leakage' (which includes all the residual energy-consuming reactions).

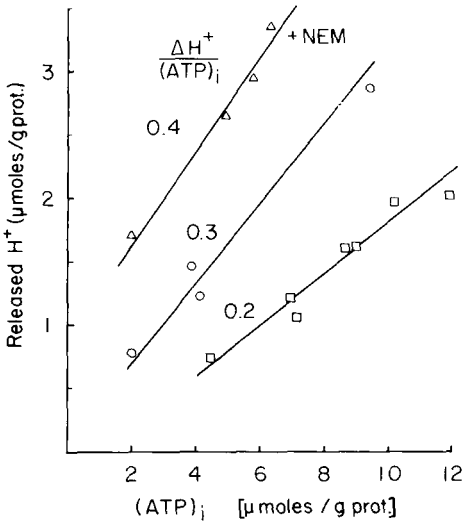


FIG. 3. The stoichiometry of H^+ release and the exchange of added ADP with endogenous ATP. Rat liver mitochondria were incubated with succinate. The amount of H^+ released is related to the amount of endogenous ATP (E. M. Klingenberg & A. Kaltstein, unpublished results). The transport of P_i was inhibited by *N*-ethylmaleimide (NEM).

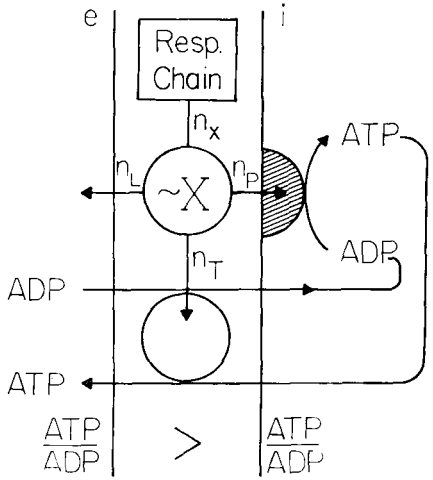


FIG. 4. The division of the respiratory energy, n_x, into the part required for ADP synthesis (n_p), for transport (n_T) and for leakage (n_L): $n_x = n_p + n_T + n_L$.

As a result of the competition between active transport and oxidative phosphorylation, the ratio of P to O should become smaller as the difference of the ATP/ADP ratios becomes higher. This relation has been experimentally

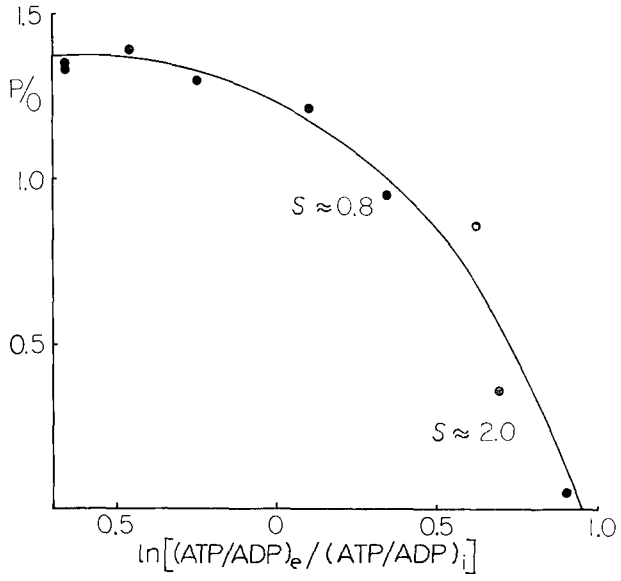


FIG. 5. The variation of the P/O ratio with the difference in the internal and external ATP/ADP ratios. Rat liver mitochondria were incubated with 0.1mM-[^{14}C]ATP, 2mM-succinate and varying amounts of hexokinase (0–1.2 units) at about 1 mg protein/ml. Uptake of oxygen and the formation of glucose 6-phosphate were measured about 2–5 min after incubation. The mitochondria were separated by silicone layer filtration and $(ATP/ADP)_i$ and $(ATP/ADP)_e$ were determined chromatographically. The amount of energy available for active transport, b, is given by $b = 2.3RT(P/O)_{max}\, S^{-1}\Pi_i^{-1} = 0.12$–$0.3$ where Π_i is the free energy of the endogenous ATP system and S is the slope of the curve.

studied by varying the external ATP/ADP ratio in a hexokinase–glucose trapping system as in Fig. 5 in which the P/O ratio is plotted against the difference in ATP/ADP ratios, an increase in which accompanies the decrease in activity of the hexokinase added. A decrease in the P/O ratio is to be expected solely on the basis of an increase of the leakage of energy; the rate of this leak should be proportional to the energy potential built up at the membrane. In the absence of hexokinase, no ATP is formed and all the energy leaks away. In these conditions, the amount of energy invested into the translocation can be varied. When the gradient $\ln[(ATP/ADP)_e/ATP/ADP)_i]$ is zero or even negative, the P/O ratio is reasonably independent of the double ratio. As the gradient becomes positive, the P/O ratio decreases more and the slope of the curve is used to evaluate the fraction, b, of the energy not recovered in ATP synthesis that is used for the extrusion of ATP (see legend to Fig. 5). This fraction ranges from 0.3 to 0.12, when the gradient is large. This decrease shows the increased significance of the leakage.

I should emphasize that in these experiments with a steady-state phosphorylation system the gradient of the ATP/ADP ratios is much smaller than in fully equilibrated systems where the ratio (r) can reach 60. Accordingly, the amount of energy invested in translocation, ΔG, can vary with the ratio across the mitochondrial membrane from 6.3 kJ ($r = 10$) to 10.5 kJ ($r = 60$).

A considerable part of phosphorylation potential of the ATP produced by the mitochondria originates, therefore, in the active transport across the membrane. The resulting differences in the phosphorylation potentials between the intra- and extra-mitochondrial spaces are probably highly significant for the development of eukaryotic cells where the coupling between the phosphorylation potentials and the redox potentials led to diverging requirements between the cytosolic glycolysis, in which the phosphorylation potential is coupled to an increase of redox potential, and the mitochondrial system of oxidative phosphorylation, in which the phosphorylation potential decreases when the redox potential of the NAD-system increases. Only the development of an energy-dependent regulated transport system for ATP and ADP will allow the coexistence and cooperation of both divergent ATP/ADP systems (Bücher & Klingenberg 1958; Bücher *et al.* 1972).

ENERGY AND TRANSPORT ACTIVITY

The first indication of an active transport was the modification of the 'apparent' specificity of the energy supply towards ADP or ATP (Klingenberg & Pfaff 1966; Pfaff & Klingenberg 1968): when the membrane is energized ADP enters preferentially whereas the uptake of ATP is strongly inhibited. This is in line with the assumption that the membrane potential is the main factor controlling the active transport by preventing the entrance of an excess of negative charges during the ATP–ADP exchange. This amounts to an apparent difference in the specific transport of ADP and ATP.

The corollary, to what extent the specificity on the inside to ADP and ATP differs in various conditions, has been re-examined. Fig. 6 compares the influx of ADP and ATP when the proportions of endogenous ADP and ATP are changed. When ADP is added, a higher exchange activity is observed and the more ATP is available for exchange. These observations indicate that, for the reverse reaction, ATP is preferred to ADP. In the uncoupled state the exchange with ADP is strongly decreased, first of all since considerably less ATP is available for the exchange. The comparison with the unenergized state with a high ADP content suggests that energy even increases the rate of influx of ADP. With ATP, the situation is reversed since here energy strongly

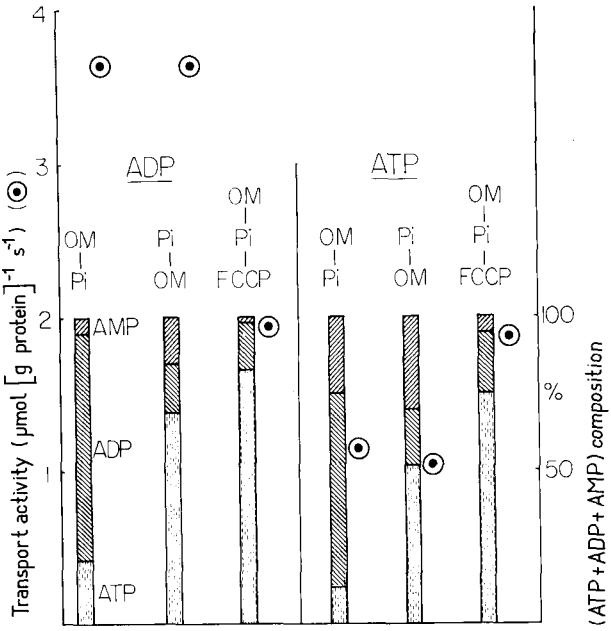
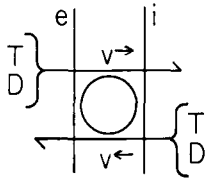


FIG. 6. Comparison of the ADP and ATP exchange with the intramitochondrial composition of ATP, and AMP in the energized and non-energized (+FCCP) states. The exchange activity is measured in rat liver mitochondria by an automatic rapid-sampling apparatus in a back-exchange system by stopping with carboxyatractylate. The endogenous proportions of ADP and ATP were changed by the incubation of the mitochondria as indicated with either first oligomycin and phosphate or vice versa. FCCP is carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone.



ΔG	Forward rates (e → i)	Backward rates (e ← i)
> 0	$V_T^{\rightarrow} << V_D^{\rightarrow}$	$V_T^{\leftarrow} > V_D^{\leftarrow}$
0	$V_T^{\rightarrow} \geqslant V_D^{\rightarrow}$	$V_T^{\leftarrow} = V_D$

FIG. 7. Scheme summarizing the energy-dependent shifts of the specificity of the rates of translocation *(v)* for ADP (subscript D) and ATP (subscript T) in the forward and backward transport reaction (shown on left hand side).

inhibits the uptake of ATP. The exchange is mainly activated on uncoupling, despite the large endogenous ADP content.

In the uncoupled state, the rate of uptake with ATP is increased but amounts to only 70% of the maximum activity of ADP in mitochondria well loaded

with ADP, a fact which indicates that the transport is still inhibited by the high endogenous content of ADP.

Fig. 7 summarizes the conclusions about the specificity of the rates of translocation. The apparent specificity for the entry depends strongly on the energization of the membrane which appears to increase the rate of entry of ADP but strongly inhibits the entry of ATP. The exit rates are largely independent of energy and are higher for ATP than for ADP.

The ratios of the rates are related to the energy invested in the active transport by equation (2). Values of the ratio in equation (2) of 20–30 give values for ΔG of -7.4 to -8.8 kJ.

$$RT \ln \frac{V_T^{\rightarrow}}{V_T^{\leftarrow}} \cdot \frac{V_D^{\rightarrow}}{V_D^{\leftarrow}} \geqslant \Delta G$$

ENERGETIC ASPECTS OF THE MECHANISM OF TRANSLOCATION

The transport of ADP and ATP across the mitochondrial membranes and, consequently, all reactions limited by the transport depend remarkably on temperature. This dependence can now be studied even for beef heart mitochondria, which have extremely rapid transport systems, with the newly developed, automatically programmed, rapid mixing and sampling techniques (see Fig. 8). The remarkably high activation energy (E_A = 134 kJ) is unusual for enzymic processes and is in agreement with the data previously reported for liver mitochondria (Pfaff *et al.* 1969).

We may gain further insight into the partial reactions responsible for the overall activation energy by following the temperature dependences of the contraction and decontraction reactions observed in mitochondria on addition of ADP. The addition of ADP to beef heart mitochondria causes a relatively rapid contraction which Scherer & I (1974) interpret as reflecting the partial movement of the carrier to the inside. The decrease in absorption on addition of carboxyatractylate corresponds to the return of the carrier outside whereupon it is trapped by carboxyatractylate after removal of the ADP (Fig. 9). Thus, the first reaction may be mainly limited by the binding rate of ADP whereas the reverse reaction may at least in part be rate-limited by the dissociation rate of ADP.

The temperature dependence of both rates has been measured in particular for the dissociation of ADP in a limited range (Fig. 10). The increase of the contraction rate is surprisingly low between 0 and 8 °C whereas the decontraction rate induced by carboxyatractylate has a higher temperature dependence (E_A = 113 kJ), similar to the overall transport. These plots indicate that the dissociation rate of ADP may be more responsible for the large temperature

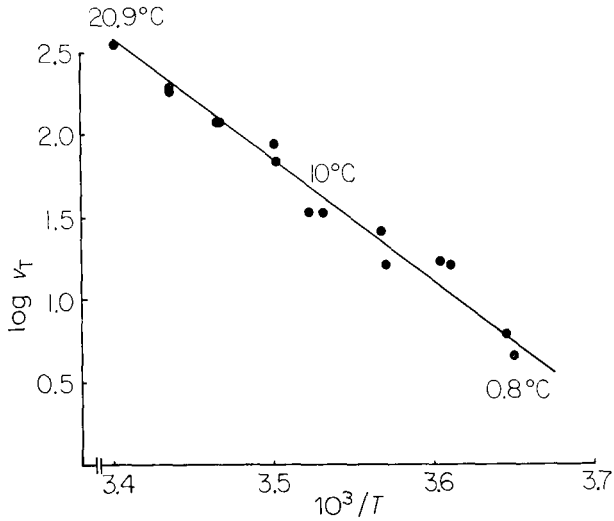


FIG. 8. The temperature dependence of the ADP transport in beef heart mitochondria: Arrhenius plot of the data obtained with a rapid-sampling apparatus. The rate was evaluated according to pseudo-first-order kinetics. The rate measurement was made by back-exchange on addition of 100μM-ADP; the mitochondria were energized by addition of P_i and substrate. The slope, the activation energy E_A, is 134 kJ.

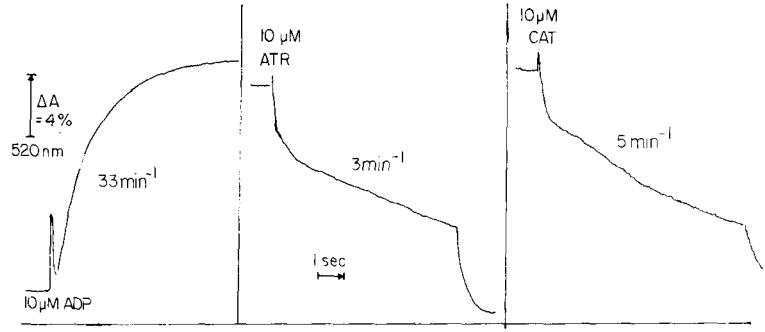


FIG. 9. The kinetics of the contraction of mitochondria by ADP and decontraction of mitochondria by atractylate (ATR) and carboxyatractylate (CAT) as measured by absorption changes at 520 nm and 8 °C. Higher time resolution was obtained by addition with a moving mixing chamber.

dependence than the binding rate. The dissociation of ADP and the transition to the empty carrier apparently require a higher activation energy than the reverse step (see later).

Another parameter possibly responsible for the large temperature dependence

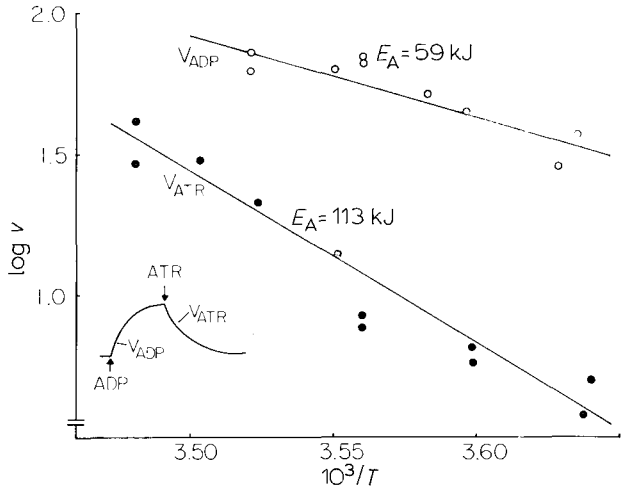


FIG. 10. Temperature dependence of the rate of contraction as induced by ADP in the mitochondria and the rate of decontraction induced by atractylate. The data are displayed in Arrhenius plots (see Fig. 8). The inset illustrates the recording of absorbance changes due to contraction and decontraction and the rate of the absorbance changes on addition of ADP and atractylate.

is the viscosity of the membrane. Bongkrekate induces a contraction that has a very large temperature dependence (Fig. 11) (Klingenberg 1975). In these conditions the slow contraction (after ATP has been added first) also results in a remarkably high temperature dependence. The hampering of the diffusion of bongkrekate by high viscosity of the membrane has been considered to be responsible for this delayed contraction.

In the model of the diffusional mobile carrier, a similar temperature dependence might influence the reorientation of the carrier through the viscosity. However, in other models such as the stationary-gate model (see later), this viscosity should play a minor role as the rate-limiting step.

ACTIVATION ENERGY AND CARRIER MECHANISM

The results enlighten the energetic problems of the transitional states of carrier action. It is, therefore, appropriate to explain the theories of carrier mechanism which have been proposed for the ADP/ATP carrier. This carrier has provided us with a unique opportunity to penetrate to the molecular level of the carrier action by virtue of the mitochondrial membrane, the high specificity to its ligand and, in particular, the existence of two highly specific

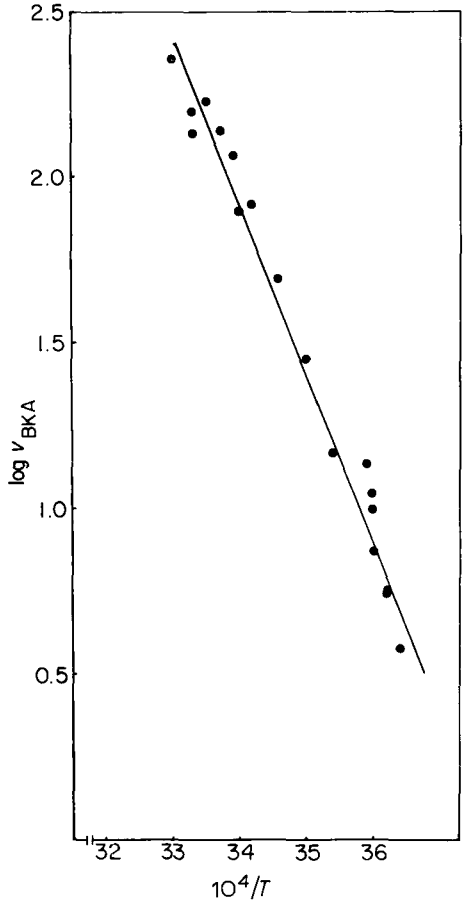


FIG. 11. Temperature dependence of the rate (v_{BKA}) of the bongkrekate-induced contraction of beef heart mitochondria. The activation energy (the slope, E_A) is 96 kJ.

inhibitors, the atractylate group of compounds and bongkrekate. Both these inhibitors are believed to immobilize the carrier on binding. Atractylate, an impermeable ligand, fixes the carrier site at the outer surface whereas bongkrekate, a permeable ligand, fixes the carrier at the inner surface (Fig. 12*A*). In the steady-state with ADP or ATP, the carrier should be equally distributed on both sides of the membrane (Erdelt *et al.* 1972; Klingenberg & Buchholz 1973; Klingenberg *et al.* 1973; Scherer & Klingenberg 1974).

The fact that all carrier sites can be brought to one or the other side with the appropriate ligand was the first demonstration of a reorientating carrier at a

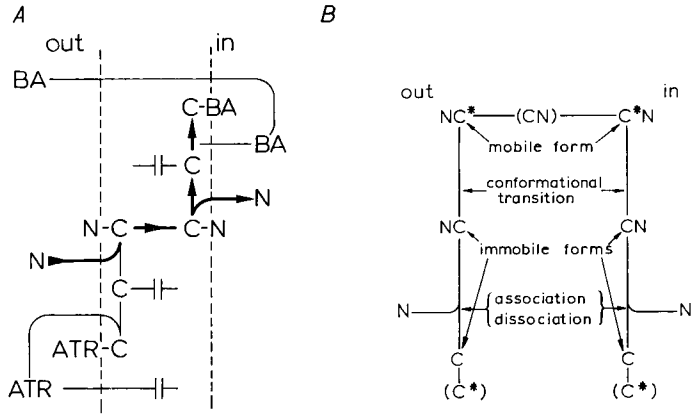


FIG. 12. (*A*) The reorientation of the carrier (C) sites: atractylate (ATR) freezes all the carriers outside and bongkrekate (BA), being able to permeate the membrane, locks the carriers on the inside. The nucleotide ADP (N) can bind to the carriers both inside and outside the membrane. (*B*) Individual steps of carrier function from association of the nucleotide with the carrier to their dissociation. C* is the mobile form of the carrier.

molecular level. Also, for a counterexchange system, we can postulate that the unoccupied carrier as well is immobile. As a result of this model, two partial steps have been postulated for the carrier mechanism (Fig. *12B*): (*a*) binding of ADP or ATP causes the carrier to pass from an immobile to a mobile state (the 'mobilizing' transition) and (*b*) reorientation of the binding site from one side to the other (i.e. by the reorienting step or the translocational transition). Then, the mobilizing step is reversed and ADP dissociates.

This mechanism can be fitted into at least two basically different carrier models (Fig. 13). In one, a mobile carrier can diffuse or rotate in the membrane, depending on whether it spans the whole width of the membrane. The reorientation consists of rotational and translational movements of the carrier. In another model, a stationary, reorienting or gated carrier—a protein—spans the impermeable width of the membrane. The binding site is oriented to either one side of the membrane or the other. When a mobilizing ligand binds, a conformational change ensues which brings the ligand into a transitional state by closing the binding site. The binding site can then open to the other side and release the ADP. The conformational changes as the result of the binding and of the transition are in this case less clearly discriminated.

In all these models the conformational changes are a key step in the carrier mechanism. The conformational changes of a carrier are probably considerably larger than for any comparable soluble protein. Consequently, energetic

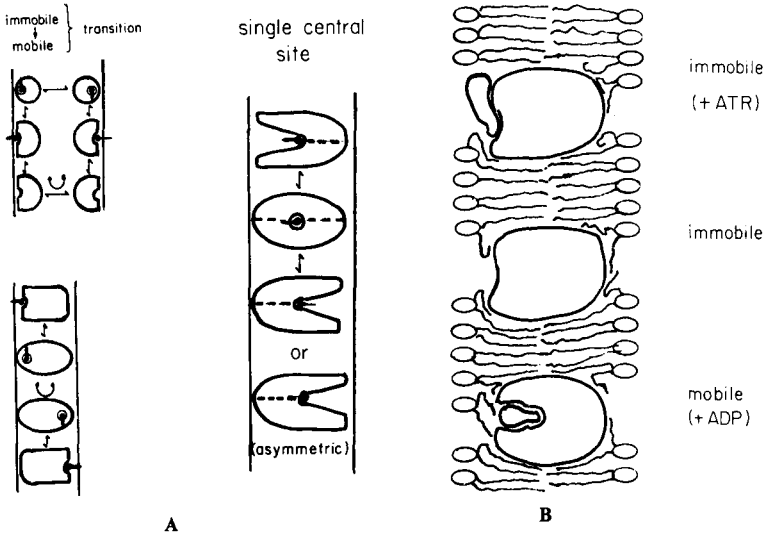


FIG. 13. (*A*) Various interpretations of the reorientating carrier model. (*B*) The carrier–phospholipid interaction under the influence of mobilizing (ADP) and immobilizing (atractylate; ATR) ligands.

aspects in understanding the carrier mechanism are of great importance. The large conformational change of the carrier should be accompanied by a correspondingly large change of its internal energy. However, this could be compensated only insufficiently by the energy released on the relatively loose binding of ADP or ATP. Therefore, I propose that it is complemented by the energy of interaction between the proteins and phospholipids. It is assumed that in the immobile, unliganded state, the carrier is open so that the interaction with the phospholipids is reduced. On binding the mobilizing ligands, the carrier becomes surrounded more by the phospholipids (Fig. 13). Binding the hydrophilic immobilizing ligands atractylate or carboxyatractylate brings the carrier into a still more open form and decreases the lipid interaction.

A scheme for the free energy balances for the binding of the three ligands is given in Fig. 14. These energy changes are only estimated to help understand the relative contribution of the different interactions. As mentioned before, the large conformational change on addition of the mobilizing ligand ADP is energetically overcome by the interaction of the phospholipids with the carrier. The conformational changes when carboxyatractylate is bound are small since the role of phopholipids is decreased and, therefore, the interaction of the ligand with the carrier is fully released as free energy, resulting in very

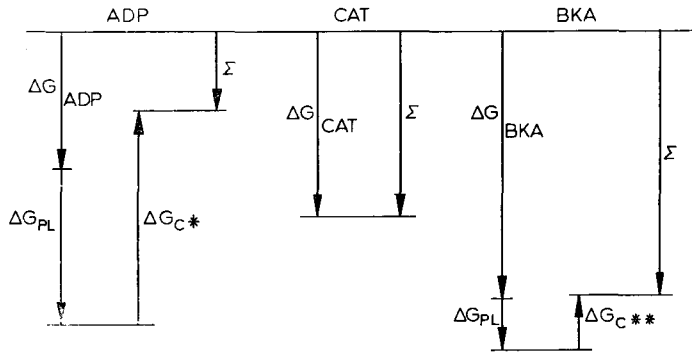


FIG. 14. Free enthalpy balance of ligand binding, of the associated conformational change of the carrier (C) and of the phospholipid (PL) interaction: CAT, carboxyatractylate; BKA, bongkrekate. $\Sigma = \Delta G_{ADP} + \Delta G_{PL} - \Delta G_C$.

tight binding. With bongkrekate also the conformational change can be considered as small and a strong binding energy is obtained.

The facilitation of the large conformational change by an increased interaction with phospholipids may be called the 'lubrication' effect of carrier activation. The phospholipid–protein interaction is a key problem in understanding the astonishing mechanism by which a carrier can transport large hydrophilic substances through a membrane. This interaction must be highly specific in order to release the energy required for this conformational change. Interaction of ADP with the carrier is a triggering mechanism that releases the energy compensation between the conformational change of the carrier and the increased phospholipid interaction. The mechanism also provides for the postulate that ADP binding at the carrier must not be too tight so that ADP may be rapidly dissociated in the overall transport.

The relatively high activation energy (E_A = 134 kJ) of the translocation may be due to interaction with the phospholipids which could have a relatively large activation energy as a result of changes in the microviscosity of the phospholipid environment. The temperature dependence of the single 'on and off' reaction, such as the contraction or decontraction, indicates that the transition to the immobilized step and the subsequent dissociation of ADP are temperature dependent rather than the binding.

Another aspect more specifically linked to the stationary gated model in contrast to the rotatory carrier model is the symmetry or asymmetry of the carrier sites when facing the different sides of the membrane. In the gated pore model, it can be visualized that on binding and releasing an asymmetric molecule

such as ADP the binding sites have different conformations on both sides (see Fig. 13), otherwise the molecules would have to rotate during the transition. The overall energy release, however, is much more easily understood in the asymmetric arrangement. Studies with sonic particles where the sides are reversed indicate an asymmetry of the membrane sites when located outside and inside. The conclusion from these studies was that the carrier, when facing the inside, has a particularly high affinity for bongkrekate and, possibly, none for atractylate. Furthermore, the asymmetry is stressed by the conclusion that a SH group vital for carrier activity may interact with *N*-ethylmaleimide only when the carrier is placed inside (E. M. Klingenberg, unpublished results).

The energization of the membrane may promote an asymmetric conformation and thus facilitate the active transport of ADP and ATP. Indications for such an energy dependent asymmetry arise from the fact that the interaction of *N*-ethylmaleimide with the SH group of the carrier is abolished on addition of FCCP (Vignais & Vignais 1972). Obviously, ADP does not succeed in activating the carrier as in energized mitochondria. The mechanism by which energization of the membrane can confer energy upon the carrier is unclear. Possibly, a group linked to the binding of ADP on the carrier is protonated and this protonation influences the conformation. As a result, the energization of the membrane can influence also the conformation of the carrier.

ACKNOWLEDGEMENT

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Discussion

Wilkie: In Fig. 1, you indicate $ATP^{4-} + H^+ \rightarrow ATP^{3-}$. Surely, in those conditions, *fractional* amounts of protons are liberated? For example, at pH 7 about 0.5 proton is released per mol of ATP hydrolysed.

Klingenberg: At the pH at which we worked (7.2–7.3), about 0.8 protons/mol are released.

Wilkie: The pH must be raised to 8.5 for the release of one proton/mol (ATP); only then is Fig. 1 correct.

Glynn: Is the 'book keeping' all right? What balances the charge?

Klingenberg: The book keeping may be variable. We can suggest a mechanism which controls whether ATP is transported electrogenically or electroneutrally. I believe that the carrier may have a dissociable H^+: $C^{3+} + H^+ \rightleftharpoons C^{3+}\cdot H^+$. The protonated form $C^{3+}\cdot H^+$, is in equilibrium with ATP^{4-} inside. This would give the electroneutral complex $[C^{3+}\cdot H^+\cdot ATP^{4-}]$. The C^{3+} form forms the negatively charged complex $[C^{3+}\cdot ATP^{4-}]$(see Fig. 1). This equilibrium, as controlled by the H^+ concentration, can influence the portion of electroneutral ATP formation.

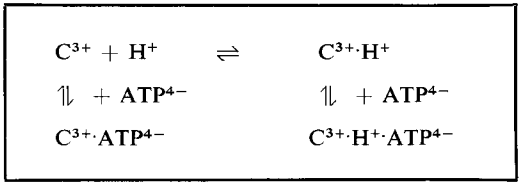

FIG. 1 (Klingenberg). Equilibria of C^{3+}, H^+ and ATP^{4-}.

Gutfreund: What worries me is that you limited the study of the activation energy to a temperature range of 0 to 20 °C. The lipids in the membranes of microorganisms can adjust themselves to the temperature in which these organisms live (i.e. viscosity transitions occur at specific temperatures), but beef heart mitochondria do not live between 0 and 20 °C. Are these high activation energies also observed at 37 °C?

Klingenberg: Most research on temperature-dependent phospholipids has concentrated on trying to find a break in activation energy. Such a break does

occur in mitochondrial reactions at 15 to 20 °C. It is remarkable that there is no freezing of mitochondrial reactions down to 0 °C (unpublished results). The mitochondrial membrane is highly unsaturated, and the degree of saturation cannot easily be adjusted. Mitochondria do not have a real phase transition in the usual sense, as has been considered in *E. coli* membranes for galactoside transport and related to phospholipid fluidity.

Gutfreund: Have you ever done experiments at 27 °C?

Klingenberg: We think it quite an achievement to measure the rates of transport of ADP and ATP in beef heart mitochondria. These rates are high, representing the most powerful carrier in all aerobic cells. In these mitochondria, the extent of transport is limited by the endogenous pools, so that such a measurement even at 10 °C for the pseudo-first-order kinetics has to be completed in two seconds. The samples must be withdrawn within a few milliseconds. This procedure is extremely difficult at high temperatures. We can decrease the reaction rate by partially inhibiting the reaction by, say, 80% and then continuing our measurements up to 37 °C.

Kornberg: Raison has reported a major difference between cold-sensitive and cold-tolerant plants (Raison 1973)—a problem of some importance for the transport of fruit and vegetables in refrigerator ships. He found very sharp breaks in the Arrhenius plot of succinate oxidation against T^{-1} with mitochondria from cold-sensitive plants, but not with mitochondria from chilling-resistant varieties. (He measured the overall rate of succinate oxidation linked to oxygen and it may be that he was actually measuring a change in the orientation of some lipid-soluble component in the membrane.) However, it isn't strictly accurate to say that these breaks have not been observed with mitochondria.

Klingenberg: Temperature breaks for several reactions can be observed in mitochondria, but most should not be compared with phase transitions in the phospholipid phase of mitochondria.

Most of these phospholipid–protein interactions are rather unspecific, so the interaction energy is probably much lower than is required in transport systems which require a much more specific interaction than in the usual 'reconstituted' systems.

Hess: What is the source of this specificity?

Klingenberg: It may originate in the particular composition of the phospholipid associated with the protein.

Roseman: A problem with these Arrhenius plots is that one is always considering the bulk solvent, which is bulk lipid. But in discussing the functional proteins in the membrane, one must concern oneself not with the bulk solvent

but with the small amount of lipid specifically combined with the protein being studied. One can never measure these specific interactions by looking at the whole system.

With regard to specificities, can AMP be transported or phosphorylated or both?

Klingenberg: The ADP, ATP transport system is very specific: only ADP and ATP are transported; dADP, dATP are transported about ten times slower and the imidophosphate derivatives show about 10% activity. The methylene analogues are inactive. All other nucleotides have no activity.

Roseman: Is the exchange of ADP for ATP 1:1?

Klingenberg: The transport is largely a 1:1 exchange. Superimposed on the exchange is a net efflux or leakage from the mitochondria; however, it is not possible in general to have a net influx. The rate of the net efflux is about 1/100 that of the exchange reaction. It probably does not have any physiological significance.

Keynes: If one labels the nucleotide part of the ATP or ADP, is the net flux of ATP equal to the flux of the labelled adenosine? In other words, is there any exchange diffusion of ADP or ATP in this carrier system?

Klingenberg: Experiments in which the base portion is labelled with ^{14}C and the phosphate with ^{32}P demonstrate that the intact molecule ATP is exchanged. There is no transphosphorylation, as has been suggested.

Hodgkin: What were the ionic conditions?

Klingenberg: We do not need to add to any bivalent ions although we cannot exclude the possibility that the carrier contains such a tightly-bound ion. Here, the activity is greater when the medium has a high ionic strength. This has been explained by the charge shielding of the highly anionic lipid surface from the approaching anionic nucleotides.

Cohn: Is there an analogy with the phospholipid–protein interaction in the mitochondrial membrane described by Gazzoti *et al.* (1974)? They isolated 3-hydroxybutyrate dehydrogenase from the membrane and showed that the phospholipid requirement for activity is highly specific. Also, NAD is bound to the protein in the presence of phospholipid.

Klingenberg: 3-Hydroxybutyrate dehydrogenase does not exhibit such a requirement for a good interaction between protein and phospholipid.

Cohn: Yet 3-hydroxybutyrate is not active nor does it bind NAD, unless mitochondrial-type phospholipid is added to it.

Klingenberg: Some analogy with the 3-hydroxybutyrate dehydrogenase exists in that the carrier does not bind ADP as soon as phospholipid is removed from the membrane. Even disturbance of the phospholipid composition of the membrane by ageing leads to a loss of the ADP/ATP binding. In contrast,

the binding of the inhibitory ligand, atractylate, is retained and insensitive to lipid disturbances.

Cohn: It might be worthwhile to determine the temperature dependence of binding in such a system.

Hess: You correlated the position of the carrier inside or outside with the degree of light scattering. Is this the only basis for your interpretation or do you also rely on binding studies?

Klingenberg: The changes in light scattering are closely related to the measured binding of ADP or the inhibitors atractylate and bongkrekate. The rates of changing in light scattering correlate well with the rate of a single turnover of translocation. However, we do not understand the mechanism of this contraction. It can be seen that the membrane surfaces are increased simply by the shifting of the carrier sites.

The movement of ATP parallels the movement of the carrier. By trapping the carrier inside with bongkrekate, exactly one molecule of ADP goes into the mitochondria. This represents a small amount compared to the total ADP content in the the mitochondria, but this can be measured. It represents the additional binding which we just discussed.

The reorientating mechanism allows several possibilities for the reorientation of the carrier sites: for example, diffusion and rotation of a mobile carrier, rotation of more fixed carrier, fixed carrier which opens the binding centre either to the inside or outside.

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Bioluminescence: from chemical bonds to photons

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Abstract The biological transformation of chemical to photic energy involves an enzyme-mediated *chemiluminescent* reaction, in which one of the products exists in an electronically excited state, emitting a photon as it returns to the ground state.

The colour of bioluminescence differs in different organisms, ranging from the deep blue (460 nm) of certain crustacea, through the bluish green (490 nm) of some bacteria, the green (530 nm) of mushrooms to the red (about 600 nm) of the railroad worm. In one case, energy transfer has been demonstrated from the enzyme system to material that emits light with a longer wavelength. The energies involved range from about 165 to 250 kJ/einstein (40 to 60 kcal/einstein).

Boyle first showed that air was involved in bioluminescence in 1668 in his experiments with an air pump. Over the past 100 years, it has become clear that most if not all bioluminescent systems require molecular oxygen. The recent isolation and characterization of an oxygen-containing (peroxide) enzyme intermediate from the bacterial system is described and a reaction mechanism is postulated. This scheme is compared with other hypothetical mechanisms, in particular those involving a four-membered ring intermediate, a dioxetane, in which the simultaneous cleavage of two bonds leaves one product in an excited state. I shall discuss the special role of luciferases in bioluminescence, especially in flashing mechanisms involving 'precharged' intermediates.

Bioluminescence, or the emission of light by living organisms, involves an enzyme-mediated chemiluminescent reaction, an exergonic oxidative chemical reaction in which part of the energy is specifically used in creating an excited state of an intermediate or product molecule. This represents a case of energy conversion *par excellence*—the conversion of chemical energy into radiant energy (Harvey 1952; Hastings 1968).

The chemistry of the step(s) leading to the population of an excited state is not yet well understood. Since the time of Boyle (1668), who with his air pump showed that the luminescence of bacteria and mushrooms could be

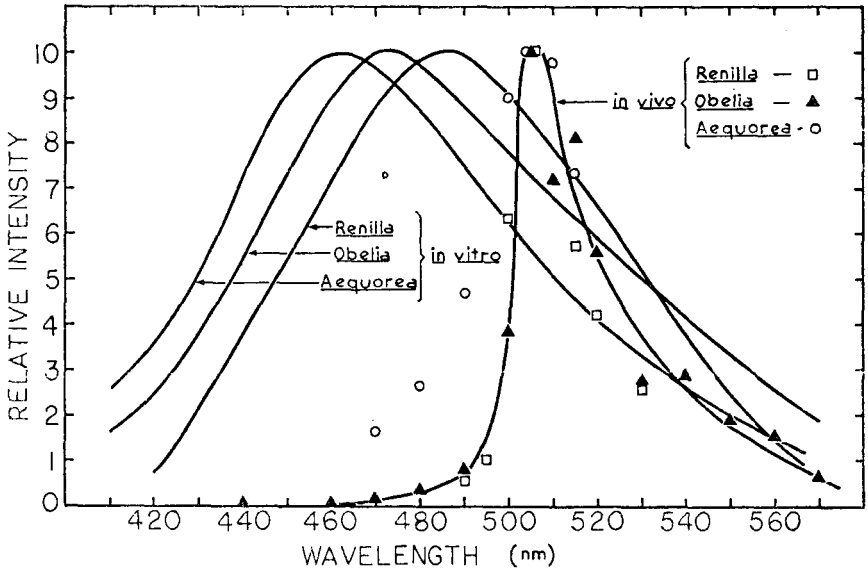

FIG. 1. Energy transfer in bioluminescent coelenterates, *Renilla*, *Obelia* and *Aequorea*. The isolated enzyme systems, or photoproteins, emit light *in vitro* on the addition of calcium with a λ_{max} in the blue. *In vivo*, all emit light with λ_{max} 508 nm, owing to the transfer of energy to a green fluorescent protein, whose fluorescence emission spectrum matches that of the bioluminescent emission (Morin & Hastings 1971*b*).

reversibly extinguished, it has been known that molecular oxygen is required in all or most systems. And it is now almost 100 years since the French physiologist Du Bois (1885, 1887) elucidated the more general nature of bioluminescent reactions. He postulated the involvement of a heat-stable substrate of low molecular weight that is oxidized—he termed this luciferin, since he presumed its oxidation product was the emitter—and a heat-labile catalyst which he termed luciferase. So, for many years a general reaction mechanism was written as in (1).

$$LH_2 + O_2 \xrightarrow{\text{luciferase}} L^* \rightarrow L + h\nu \qquad (1)$$

Though this general formulation is valuable, it has limitations. First, the luciferins from different organisms are structurally completely different, as are the specific luciferases. So the terms luciferin and luciferase are generic and refer to no specific molecular species, or even class of molecules, chemically speaking.

Secondly, as might be expected, the generalized formulation does not apply

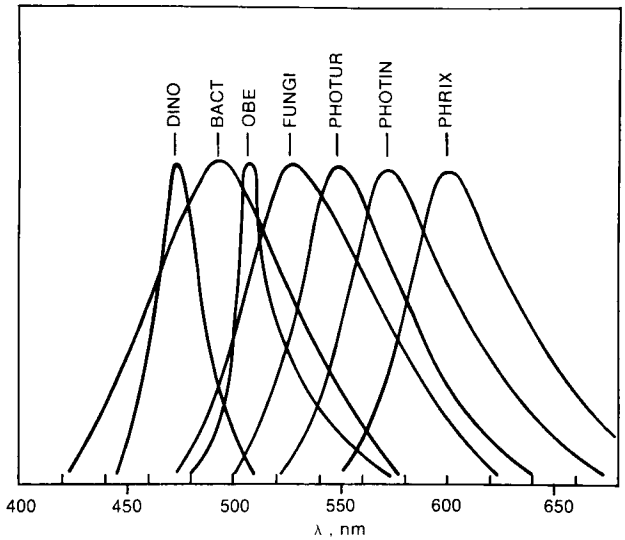

FIG. 2. The characteristic emission spectra of some bioluminescent organisms, showing that they emit at many different wavelengths. This also illustrates large differences in wavelength between the emitted light of two genera of fireflies (*Photuris* and *Photinus*): DINO, *Gonyaulax polyedra*; BACT, *Photobacterium fischeri*; OBE, *Obelia geniculata*; FUNGI, *Omphalia flavida*; PHOTUR, *Photuris pennsylvanica*; PHOTIN, *Photinus scintillans*; and PHRIX, *Phrixothrix* sp.

in detail to most systems, including two groups of organisms which Du Bois studied in some detail. For example, Michelson and his co-workers showed that in the clam *Pholas* the substrate is a heat-labile protein of high molecular weight (Henry *et al.* 1970; Henry & Michelson 1970). In the fireflies, which Du Bois also studied, the luciferin is not oxidized directly. First, it reacts with ATP to form the adenylate which is then oxidized with the emission of light. A comment often heard, to the effect that ATP provides the energy for light emission, is thus obviously incorrect.

The energies involved are considerable with respect to those usually encountered in biological systems; a photon at 500 nm has an energy of about 50 kcal/einstein (210 kJ/einstein), so the reaction must be highly exergonic. Recent calorimetric determinations confirm this (Langerman 1974).

In most organisms the wavelength of the emitted light in an *in vitro* system is the same *in vivo*. In the coelenterates, however, we have recently shown that the *in vivo* bioluminescence of many species involves energy transfer (Fig. 1). From the excited product molecule of the calcium-activated photoprotein, which characteristically emits in the blue, energy is transferred to a green

fluorescent protein, with emission at 508 nm. Although all the luminescent coelenterates studied contain photoproteins, not all of them have the green fluorescent protein; in these cases, the emission *in vivo* is directly in the blue.

The wavelength of the bioluminescent light differs from species to species, a fact presumably related to the function of the light emission in each organism (Fig. 2). The colour ranges from the deep blue (460 nm) of certain crustacea through the blue green (490 nm) of bacteria and the green (530 nm) of mushrooms to the yellow (560 nm) of fireflies and the red (600 nm) of the railroad worm. At the red end of the spectrum, the energy of the photon is about 40 kcal/einstein and for the light emitted in the blue it is nearly 60 kcal/einstein.

Where does the energy come from? What kind of chemical process is it that releases enough energy in one step to populate an excited state? Two different generalized schemes are now considered for chemiluminescence in liquid phase: electron transfer and cleavage of ring peroxides.

The first is evidently the simpler, with the transfer of one electron from a donor radical anion to an acceptor radical cation, or the oxidation of this electron acceptor by some oxidant (Fig. 3) (Faulkner & Bard 1975). Such an electron transfer should be a very fast process, during which the structure of the participants does not alter, thereby maximizing the energy available for electronic excitation. Although more and more examples and better knowledge of the mechanism are now available for such reactions in chemical and electrochemical systems, such a mechanism has not been proposed for any bio-

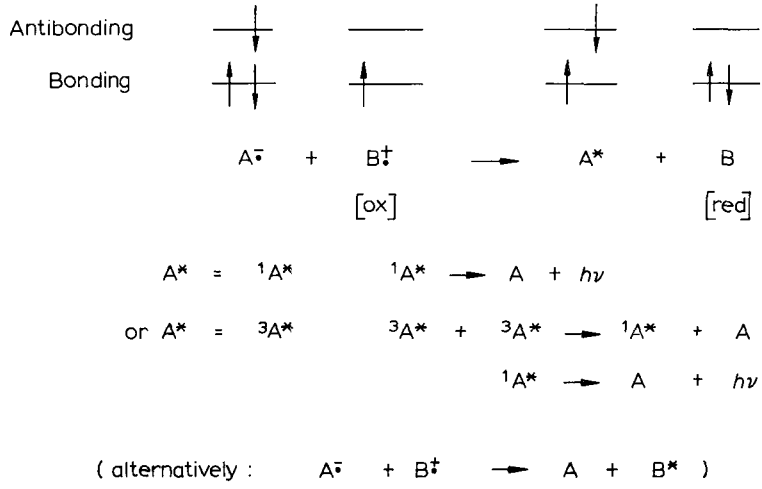

FIG 3. Hypothetical mechanisms of electron transfer for chemiluminescence.

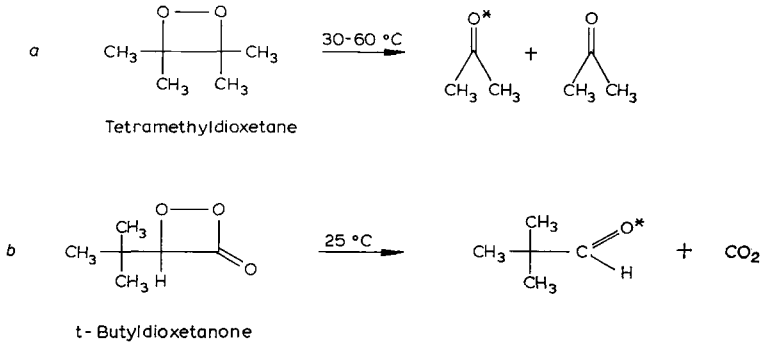

FIG. 4. Hypothetical mechanisms for the cleavage of simple dioxetanes (*a*) and α-peroxylactones (*b*). The excited products decay, emitting light; hence, the chemiluminescence.

luminescent reactions. This may be due in part to the distraction we have suffered from as a result of the ubiquity and apparent importance of the oxygen molecule itself and our reluctance to think in terms of a single electron.

Considerable circumstantial evidence now exists for the participation of four-membered-ring peroxides (dioxetanes and α-peroxylactones) (Fig. 4) in several bioluminescent systems, such as the firefly (Fig. 5) and *Cypridina*

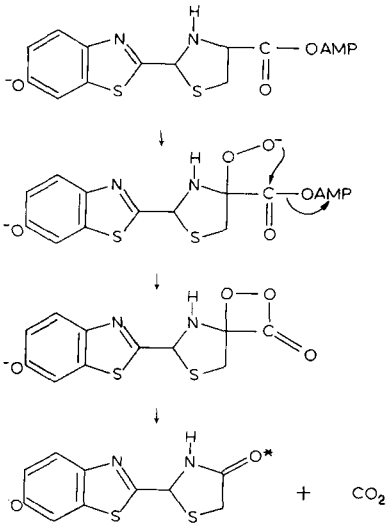

FIG. 5. Fireflies emit light as brief (~0.2 s) bright flashes within a light organ whose reactants are mobilized in an unknown fashion. The biochemical events are better understood in the isolated system; the luciferin molecule (top) is oxidized in two distinct steps: first, reaction with ATP to form an adenylate followed by oxidation of this molecule. A postulated pathway with a dioxetane intermediate is shown.

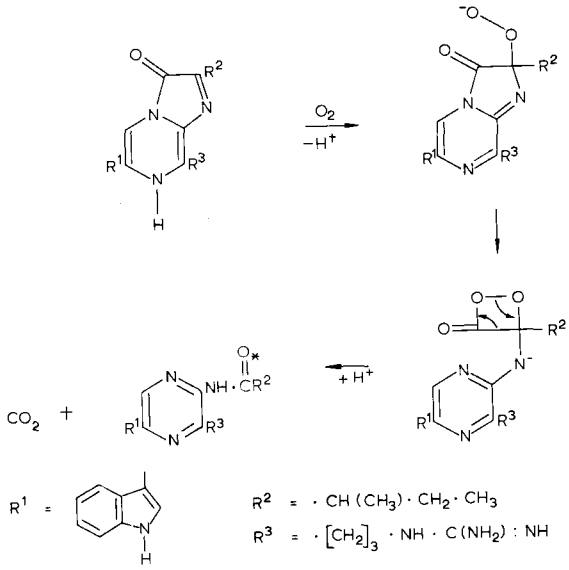

FIG. 6. The luciferin from *Cypridina* and the postulated reaction mechanism for chemiluminescence with a dioxetane intermediate. *Cypridina* is a small crustacean which synthesizes its luciferin and luciferase in two separate glands and then squirts the two compounds into the sea water where they react to produce a flash of light.

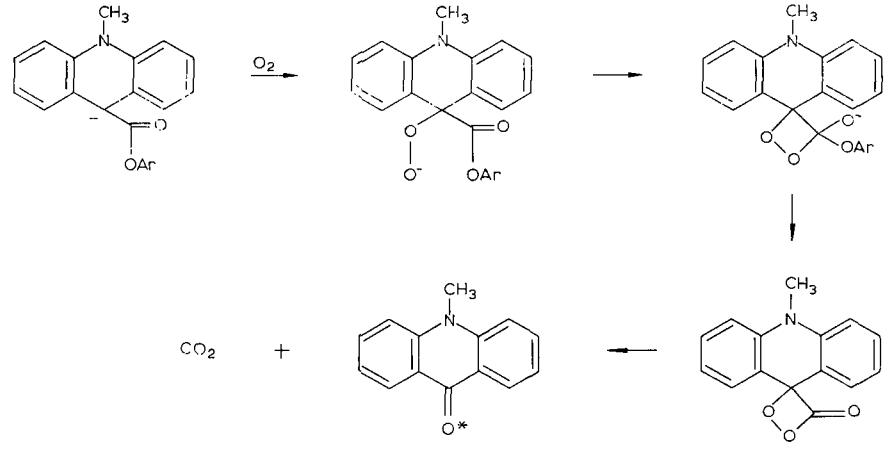

FIG. 7. A mechanism proposed for the chemiluminescent autoxidation of acridine esters in strong base (McCapra 1973). The quantum yield of the reaction, Φ, is 10%.

(Fig. 6) (McCapra 1973). Fig. 7 shows the reaction mechanism for the autoxidation of a chemiluminescent acridine ester proposed by McCapra (1973). The dioxetane compounds, postulated as intermediates in these reactions, are supposed to undergo thermal cleavage readily, yielding CO_2 and an electronically excited carbonyl product which emits a photon.

Considering the weakness of the peroxide O–O bond, the strain of the four-membered ring and the large amount of energy gained by the formation of the two carbonyl double bonds, we can easily see that the decomposition of these compounds should generate a great amount of energy—roughly 100 kcal—more than enough to account for the photon observed.

Although the large dioxetanes and α-peroxylactones hypothesized in Figs. 5, 6, and 7 have not yet been isolated, some simple prototypes of these structures have now been synthesized (Fig. 4). Surprisingly, most of the dioxetanes made so far, with methyl, phenyl or other small substituents, are remarkably stable; they have lifetimes of the order of days or even years at room temperature. (The α-peroxylactones are the least stable with lifetimes shorter than an hour.) Yet when they decompose on heating, a considerable fraction of the carbonyl fragments generated (perhaps 25%) are electronically excited, as expected. Surprisingly, however, these excited molecules appear to be overwhelmingly in triplet rather than singlet states (Lee & Wilson 1973; Turro *et al.* 1974).

No agreement has yet been reached about the theoretical explanation of these reactions; the controversy between the advocates of a concerted decomposition and those who favour a biradical intermediate (after rupture of the O–O bond) is likely to continue until more experimental facts are available (O'Neal & Richardson 1970; Kearns 1971; McCapra 1973; Turro *et al.* 1974). One would expect, for example, that more drastic substitution of the dioxetane would result in singlet rather than triplet products, and hence truly chemiluminescent systems. It is worth pointing out that the efficiency (~30%) of some chemiluminescent reactions now known is comparable to that of the bioluminescent systems; in other words, enzymic processes no longer have a monopoly of high quantum yields.

The bioluminescent system from luminous bacteria apparently constitutes a case in which dioxetanes do *not* participate in the mechanism. This leaves one with the thought—but no evidence—that electron transfer might be the mechanism. In these bacteria, the luciferin is one component of the electron transport chain, namely, riboflavin 5′-phosphate in its reduced form ($FMNH_2$). For light emission, another substrate is required—a long chain aldehyde which is oxidized to the corresponding acid (Shimomura *et al.* 1972). The postulated mechanism requires the concomitant oxidation of the two substrates (reaction 2), with emission of light at 490 nm.

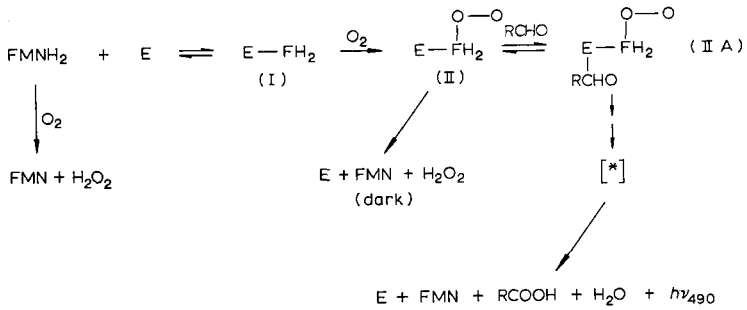

FIG. 8. Hypothetical scheme for the reactions *in vitro* in the system catalysed by bacterial luciferase (E). The reduced flavin mononucleotide may be oxidized both non-enzymically and enzymically. If the oxidation of the luciferase–flavin intermediate ($E–FH_2$) is coupled to the oxidation of aldehyde, an excited state (*) is produced.

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + h\nu_{490} \quad (2)$$

The luciferase, now available pure in gram quantities, is a dimer of molecular weight 79000 comprising two non-identical subunits, α (mol. wt. 42 000) and β (mol. wt. 39000) (Friedland & Hastings 1967*a*,*b*; Gunsalus-Miguel *et al.* 1972). Isolated subunits are inactive but when recombined they recover full activity. The function of the individual subunits can be studied in several ways, including the use of mutant luciferases with lesions in one or the other subunit. The α subunit appears to be the catalytic subunit; the specific function for β is not yet known (Meighen *et al.* 1971*a*,*b*; Cline & Hastings 1972). Studies in progress indicate that the α and β units are homologous and, possibly, represent a case of gene duplication, so that the two dissimilar subunits continue to function together as a single protein.

As indicated in equation (2), this luciferase catalyses the oxidation of reduced flavin with oxygen as the acceptor, but this reaction can proceed in either the uncoupled (dark) or coupled (light-emitting) mode (Hastings & Gibson 1963; Hastings *et al.* 1966*a*). In the coupled reaction, the long-chain aldehyde is oxidized at the same time in a reaction like that catalysed by a mixed-function oxidase. The overall scheme as presently postulated (Hastings *et al.* 1973*a*; Nicoli *et al.* 1974) is depicted in Fig. 8.

The reaction is unusually slow; the turnover time (10–20 s) is long for an enzyme-catalysed reaction (Fig. 9). The apparent catalysis of the oxidation of $FMNH_2$ in the luciferase pathway is slower than the uncatalysed pathway by a factor of almost 100. The practical consequence is that, after the enzyme is mixed with the substrate, the lifetime of the enzyme intermediate is about 100

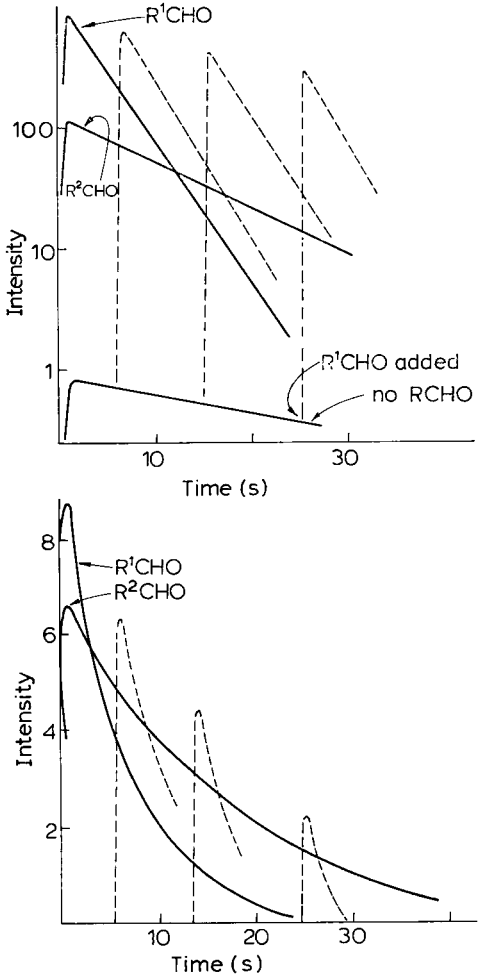

FIG. 9. The time course for bioluminescent reactions at 20 °C illustrating the lifetime of the intermediate(s), its exponential decay (— in upper graph), the low emission in the absence of aldehyde, the stimulation by aldehyde which is effective even when added later in the reaction (– – –) and the fact that different aldehydes (e.g. R^1CHO and R^2CHO) may influence the apparent first-order rate constant decay of the intermediate(s). Upper graph, light intensity in arbitrary units plotted on a logarithmic scale. In lower graph, the intercept of dotted lines with the abscissa indicates the addition of R^1CHO.

times longer than that of the substrate. In effect, one can offer the enzyme a pulse of substrate and then look at the intermediate(s) which are formed and later emit.

The exact value for the turnover time may differ with added aldehyde; it

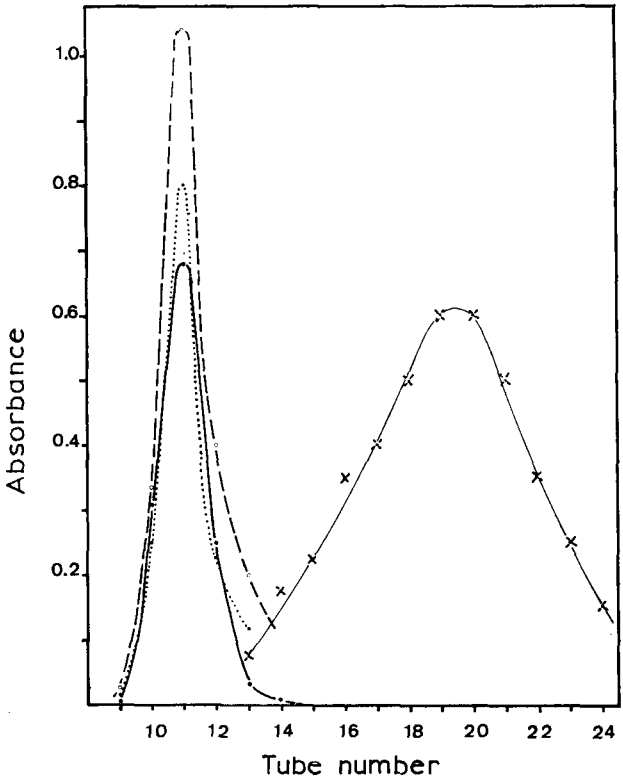

FIG. 10. Chromatographic separation of the luciferase–flavin intermediate on Sephadex LH20 at –20 °C. The free oxidized flavin (×; 450 nm) is well separated from the protein peak (------; 280 nm); the latter coincides with the bioluminescent activity (———) and a species absorbing at 372 nm (. . . .). We assayed aliquot portions from each tube for bioluminescence by injecting 0.1 ml into phosphate buffer (2 ml), pH 7, at 22 °C with 50μM-dodecanal.

differs somewhat with aldehydes of various chain lengths between C_8 and C_{18} (Hastings *et al.* 1963; 1969*a*). However, the formation of the intermediate does not require aldehyde (Figs. 8 and 9), and the lifetime of (II) (Fig. 8) is similar to that of the intermediate in the presence of aldehyde (IIA). Addition of aldehyde to a reaction initiated without it results in the prompt shift of the intermediate from (II) to (IIA). The quantum yields of (II) and (IIA) differ greatly. The small amount of light which is emitted in the absence of added aldehyde (10^{-2} to 10^{-4} of that in its presence) may be due either to contaminating aldehyde or to the fact that the dark pathway is not truly dark, but simply of low quantum yield.

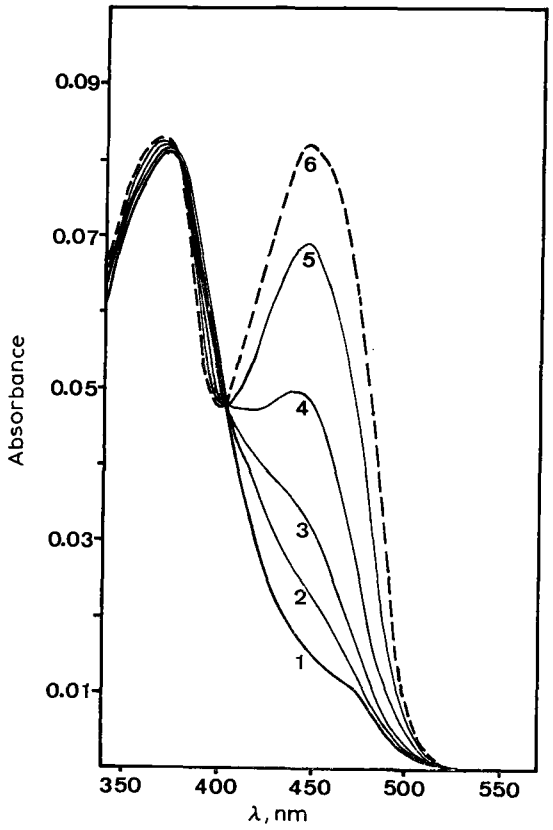

FIG. 11. Optical absorption of the peroxy intermediate (II) (see Fig. 8) at −20 °C (1) and its conversion into product (FMN) on warming to +20 °C (6). The spectra (2–5) were recorded at temperatures between −20 + 20 °C.

As the first enzymic steps for the addition of oxygen are rapid compared to the later ones, we have been able to isolate an intermediate in stoichiometric amounts and, using low temperature technology, to purify and characterize it (Hastings *et al.* 1973*b*). We initiated the reaction by mixing $FMNH_2$ and luciferase in 50% ethylene glycol–phosphate buffer (with oxygen) at pH 7 and about 5 °C, and after five seconds cooling the mixture to −20 °C or lower temperatures. We then chromatographed the material on Sephadex LH20 at −20 °C to separate the high molecular weight fraction from all the low molecular weight material, such as FMN and H_2O_2 (Fig. 10).

If the intermediate (II) behaves as shown in Fig. 8, it should bioluminesce simply on injection into a buffer containing aldehyde at +20 °C; this is so,

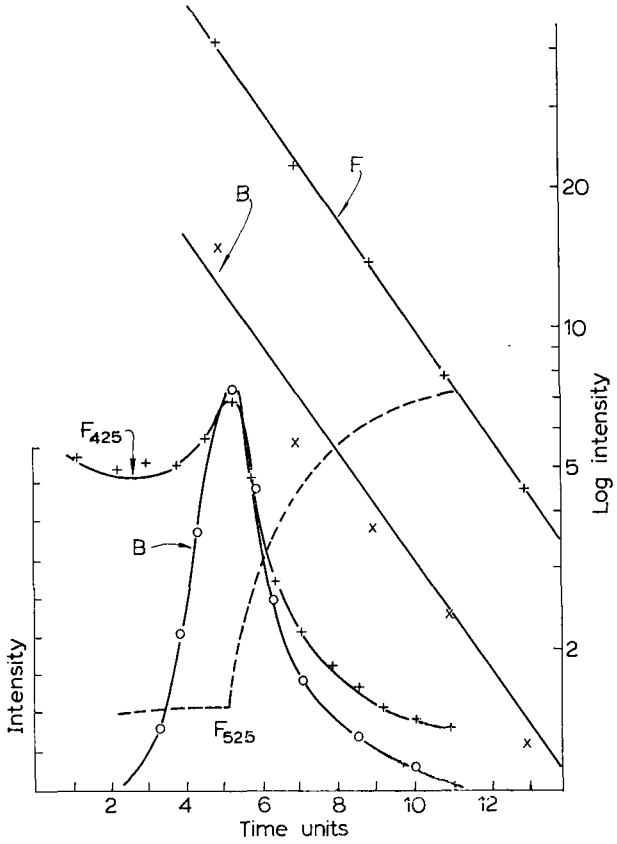

FIG. 12. Fluorescence (F) and bioluminescence (B) of the intermediate (II) on warming from −30 to +20 °C, plotted as a function of the time during which the temperature was raised. The three curves at lower left (B, F_{425}, F_{525}) are plotted with intensity on a linear scale (left, arbitrary units). On the abscissa (time), one unit equals 50 s for these curves but 25 s for the two curves (top right: B,F) plotted on a logarithmic scale for light intensity. As the temperature increases (at 3 units, $T = -15$ °C; at 4, $T = -5$ °C; at 5, $T = +5$ °C; at 6, $T = +10$ °C) the apparent fluorescence (F; excited at 375 nm, emission at 485 nm) increases, owing to the bioluminescence (B, on a more sensitive scale). Both fluorescence and bioluminescence decline exponentially (two top right-hand lines). At the same time, the fluorescence at 525 nm (F_{525}; excited at 450 nm) increases.

and the activity so obtained coincides with the protein. The first-order rate constant for the decay of the bioluminescence from this intermediate is independent of its concentration, but influenced (see Fig. 9) by the chain length of the aldehyde.

The absorption spectrum of this intermediate coincides with that of neither

the reduced nor the oxidized flavin: it absorbs with a single maximum at 373 nm (Fig. 11, 1). From the scheme of Fig. 8, we can predict that the reaction will go to completion by a dark pathway simply on warming. The spectral behaviour as the temperature is raised from –20 to +20 °C (Fig. 11) confirms this: at intermediate temperatures, mixtures of the luciferase intermediate and the product FMN are observed. Equimolar amounts of H_2O_2 and FMN are produced. By way of the light-emitting, coupled (aldehyde), pathway H_2O_2 is not produced but the amount and spectrum of the FMN formed are the same as by the dark pathway. The photon yield from the luminescent pathway was about 0.15 photon/mol FMN produced; since the fluorescent quantum yield of FMN is 0.3, this value indicates either that the chemiluminescent steps are 50% efficient or that the fluorescent quantum yield of the emitter is 0.15 (or some other combination of these factors). The specific activity of the intermediate, also calculated on the basis of its flavin content, is slightly greater than the best obtained by the usual assay, which may owe to the fact that the usual determination is quoted in terms of the activity per unit protein. The ratio of flavin to protein in the intermediate was 1 to 1.6 in the early experiments; more recently, values of 1 to 1.3 have been obtained. Additional and more rigorous determinations of this ratio are needed.

This intermediate has a relatively weak fluorescence, excited at about 375 nm. The fluorescence emission is in the range 480–520 nm, apparently involving more than one species. On continued exposure of the intermediate(s) to light, the intensity of the fluorescence increases by a factor of 5 or more and its spectrum shifts to that of an apparently single component with an emission maximum at about 485 nm (uncorrected for phototube sensitivity). On warming, this fluorescence is lost and the fluorescence of oxidized flavin mononucleotide (FMN) appears (525 nm); if warming is carried out in the presence of a long chain aldehyde, bioluminescence occurs during the transition (Fig. 12). The emission spectrum of the bioluminescence, with a peak at 485 nm, corresponds exactly to the fluorescence emission spectrum of the intermediate formed by irradiation. This correspondence suggests that the latter may be structurally similar to the emitting species in bacterial bioluminescence.

These observations are consistent with the postulate that the intermediate is an oxygenated reduced flavin bound to the luciferase (Fig. 8). More specifically, Eberhard & I (1972) proposed that the oxygen adds at the 4a position to give the peroxy anion (Fig. 13). It would be attractive to have a mechanism modelled on the firefly and *Cypridina* systems, with a four-membered-ring intermediate. McCapra & Hysert (1973) put forward such a scheme in which aldehyde adds to the flavin ring before the reaction with oxygen. However, all

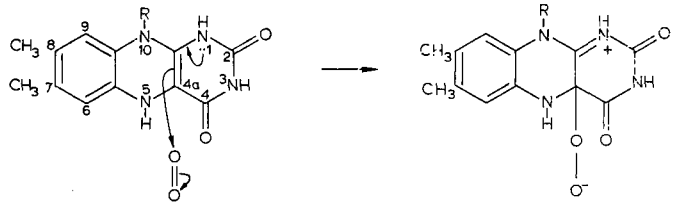

FIG. 13. Hypothetical reaction of oxygen with reduced flavin mononucleotide, bound to the luciferase to give the intermediate (II).

evidence indicates the oxygen adds covalently before the aldehyde, so this particular scheme seems untenable. Our mechanism (Eberhard & Hastings 1972) seems consistent with present observations and energetically reasonable, but it suffers from the requirement that several bonds must be made and broken simultaneously at the last excitation step.

The present knowledge of the chemistry and enzymology in several other bioluminescent systems is instructive. In many, the display of luminescence occurs as a brief bright flash. The ways in which such flashes are evoked and controlled are not understood and, although details are probably different in different systems, there may be some points of general interest. For example, colourless proteins of molecular weight about 25 000, which have the capacity to emit light, have been isolated from coelenterates (Shimomura *et al.* 1962): one has only to add calcium for luminescence with a good (0.1–0.2) quantum yield. Neither oxygen nor any other reactant is required or involved: only protein and calcium. These proteins have been referred to as photoproteins and have been isolated from many different species (Morin & Hastings 1971*a*). Such systems were termed precharged systems (Hastings 1968) in view of the fact that such proteins are formed in the cell and stored before use. Clearly, analogy with the long-lived intermediate of the bacterial system is appropriate. The reaction in coelenterates occurs within some cell particle or compartment. In the absence of calcium, the intermediate has an indefinitely long lifetime in these conditions, and then the mobilization of calcium provides a trigger to allow the reaction to go to completion with light emission (Hastings *et al.* 1969*b*; Hastings & Morin 1969). If we can relate the mobilization of calcium to an electrical event at the membrane, we can relate flashing to a membrane action potential.

Thus, we do *not* have a mechanism involving substrate storage which is then mixed with an enzyme. The stored molecule is, instead, an enzyme–substrate

intermediate, and the light emission is associated with a protein-bound excited state. Here, again, we see that the luciferase escapes a conventional catalytic role, taking on instead a substrate-like role. A 'package' of molecules poised to provide a flash of photons is a package of proteins not a pool of substrate being operated on by a catalyst rapidly turning over.

Going back to the bacterial system, we can view the aldehyde as being analogous to calcium in a restricted sense; if the intermediate were more stable in the absence of aldehyde the analogy would be better.

Conditions were once reported (Hastings & Gibson 1963) in which the intermediate was *much* more stable in the absence of aldehyde and, although we have not been able to duplicate these, such conditions may exist *in vivo*.

Still another and interestingly different example comes from a group of unicellular organisms, the dinoflagellates—algae which are ubiquitous in the oceans and give rise to the so-called phosphorescence of the ocean, the tiny sparks of bioluminescence which are seen at night when the water is disturbed by a fish, a paddle or a swimmer. The biochemical basis for this luminescence—or more particularly for its control—is most unusual. Leaving aside many of the details (see Fogel & Hastings 1972; Hastings *et al.* 1966*b*; Henry & Hastings 1974; Krieger *et al.* 1974), we can extract from the cell at pH 8 particles of a reasonably large size (mol. mass about 10^9) from which a flash of light can be obtained simply by lowering the pH from 8 to 5.7 in the presence of oxygen (DeSa *et al.* 1963). No other factor is required, and kinetics of the emission by this *in vitro* system closely resemble the *in vivo* flash seen on mechanical stimulation.

In the postulated mechanism for this system, the particle membrane includes two specific proteins, one a protein binding luciferin and the second a luciferase. The former binds luciferin at pH 8 but not at pH 6 and shelters it from reaction when bound. The luciferase binds luciferin at pH 6 and not at pH 8, and 'catalyses' its bioluminescent oxidation at pH 6. In the poised or precharged state of the membrane, it is proposed that the enzyme is localized adjacent to one or several precharged substrate carrier proteins; on a pH change, which is the membrane-tied trigger, luciferin is released and each enzyme molecule receives a single substrate molecule. The ensemble releases a flash derived from the concerted reaction of many or all the luciferases operating, in effect, in synchrony. The duration and kinetics of the flash are determined by, and identical to, the turnover time (i.e. single catalytic cycle), which in this case is about 0.2 s, still relatively long.

Why is this so? Why is it that the turnover rate is so slow, relatively speaking, in many bioluminescent systems? Is there a chemical reason—perhaps, the existence of some common and inescapable intermediate compound whose

inherent lifetime dictates this—perhaps even a triplet excited state? It seems unlikely that a triplet state is an intermediate.

Might the long lifetime be for reasons of energy utilization? Could intermediates in bioluminescence be used in certain cells in some biochemical reactions? Excited states *are* utilized by cells in photosynthesis and vision, etc., so possibly there exist, specific, if perhaps highly specialized, cases of utilization of self-generated excited states or the precursors thereof, as in photoxidation, for example (Hastings 1968). As noted before, bioluminescent systems are potential sources of substantial energy, considerably greater than that available from most other biochemical reactions. Accordingly, might the long lifetime of an active intermediate mean that it would be available to the cell longer to participate in this chemistry? This seems possible, but a specific model is not easy to formulate.

Another possibility is that the long turnover has been selected in evolution as a physiologically significant mechanism that has potential in terms of controlling the size of the photon burst, its synchronization and duration. This seems plausible; the duration of a flash in which there is a rapid turnover (compared to flash duration) depends to a great extent on the exact concentration of the catalyst or enzyme. The turnover time of a catalyst, however, is invariant, irrespective of its concentration. In firefly communication, the exact duration of the flash has important meaning. This may be so in other cases.

So, the flash duration may be tied to turnover time. The *size* (intensity) may also be related to this. As a consequence of their slow turnover, luciferases cannot be cast in the conventional role of being able to process large quantities of substrate in a short period of time. A different and novel function of luciferase in the precharged systems is that it may be enlisted in a truly synchronized emission, a laser-like burst of activity, in which all units are engaged at once and from the same starting time (Henry & Hastings 1974). In such conditions each luciferase is working at its true V_{max}, even though the substrate reacts stoichiometrically with the enzyme. Such a function seems interesting and relevant for the flashing emission of light, since the brightness of the emission does not depend on the total number of photons emitted but on the instantaneous rate of reaction.

ACKNOWLEDGEMENTS

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Discussion

Hess: Is the quantum yield only 10% or does the low value reflect a problem of standardization?

Hastings: The quantum yield is almost certainly within a factor of two of the value quoted; the uncertainty lies in the standardization, which up to now has only been done in two laboratories. The apparent low value should not be viewed as an energetic deficiency but as an efficiency in populating and quenching

excited states. Fluorescent molecules, even those which are regarded as bright, may have only fractional fluorescent quantum yields, for example, 0.3 for flavin mononucleotide. On the assumption that all the other processes taken together are only 30% efficient, the overall yield is about 10%. Bioluminescent systems do exist with higher yields, notably the firefly system *in vitro*, for which the yield is close to 1.0 (Seliger & McElroy 1960).

Taylor: Are these *aqueous* non-enzymic systems that have high quantum yields?

Hastings: No, they are mostly non-aqueous. However, aqueous systems with yields up to 1–3% have been reported (White & Brundrett 1973).

Gutfreund: How does the accurate measurement of light for this kind of system differ from that used to determine the quantum yields of rhodopsin?

Hastings: With rhodopsin one measures the absorption of light but in luminescence one measures the emission of light. However, the techniques and associated difficulties are much the same.

Hagins: In attenuating a bright light to match it to bioluminescence, we introduce calibration errors. For example, an error of 5% in the absorbance of one filter may account for a factor of 2 in transmitted flux if, say, 20 filters are stacked together.

Hastings: Again, I don't think we should attach too much importance at the moment to quantum yields in bioluminescence.

McClare: Have you applied the same approach to bioenergetics that you are applying so successfully here? For instance, the protein aequorin exists in a high energy state. Addition of calcium triggers the emission of light (which is a useful form of energy because it is visible and, for example, can activate a photocell). Imagine similar events with infrared radiation instead at around 3 μm wavelength or 40 kJ/einstein. This is the order of the energy in transformations that are interesting in bioenergetics.

Hastings: Although biochemical systems are known that emit visible light, the intensities, and probably the yields, are low. Also, the specific reactions underlying the bioluminescence are generally not known. As yet, we have no suitable detectors for emissions of low intensity, but such emissions may exist.

McClare: In Fig. 3, you depict an oxidation–reduction by charge transfer in an excited state. I have suggested (McClare 1974) that such an excited state in the i.r. region might result from such a process—perhaps, for instance, during the formation of the charge-transfer complex between NADH and FAD. Such excited states are theoretically possible. Applying the same photochemical reasoning as you applied in your paper but with i.r. energies to bioenergetics, I suggest that we could explain all these problems. For example, if we postulate that ATP hydrolysis produces an i.r. excitation—a bond vibration perhaps—then

the production of quanta of the same frequency in some other way should facilitate the synthesis of ATP while the use of the quantum for some other purpose—working a muscle (McClare 1972*a*,*b*), for example—should allow the use of ATP.

Huxley: Is this a distinction between 'conceivable' and 'known'?

McClare: Yes, but not so far in living systems. The junction between an *n*-type and a *p*-type semiconductor produces the equivalent of a charge-transfer complex and in so doing i.r. radiation is emitted. If the movement of charge is maintained with a current, then a continuous beam of radiation results (it is also coherent). In effect, I am suggesting that living systems employ the same principles as solid-state devices like this; this is not a new idea.

Wilkie: Is oxygen involved in the aequorin reaction?

Hastings: Absolutely not. I believe that the active species is a dioxetane or a dioxetane precursor like a peroxide which already contains oxygen.

Wilkie: So, light can be emitted completely anaerobically?

Hastings: Yes. However, other coelenterates (such as *Renilla*) contain an enzyme system which requires oxygen. Maybe such a system also exists in *Aequora* but simply has not been detected.

Whatley: Do the excited molecules always adopt a triplet state?

Hastings: No. Triplet states have not been implicated in any biological system, but they have been observed as intermediates for simple dioxetanes and α-peroxylactones. In all bioluminescent systems and the more complicated dioxetanes, the singlet state is populated directly.

Hodgkin: The ecological significance of light emission has always baffled me. It is all very well to talk about larger animals finding their mate but coelenterates do not mate in that way. The quantity of light emitted by micro-organisms is huge. What is it for?

Hastings: Not so much energy is expended in light emission as might be supposed from its intensity: it is our photoreceptors that are sensitive. In bacteria, the energy for bioluminescence accounts for about 1% of the total energy used. Moreover, the luciferase in bacteria is inducible rather than constitutive; they produce the inducer themselves, so only when they are growing in confined conditions in which the inducer will not diffuse or be washed away will the luciferase be made. This condition implies a specific ecological solution associated with luminescence.

Luminous bacteria live as symbionts with many species of fish which culture them on chemostat-like organs. Fig. 1 depicts such a fish, *Photoblepharon*, which lives on the coral reef in the Gulf of Eilat. The fish possesses organs in the orbit just beneath the eye; the light can be controlled by lids which move

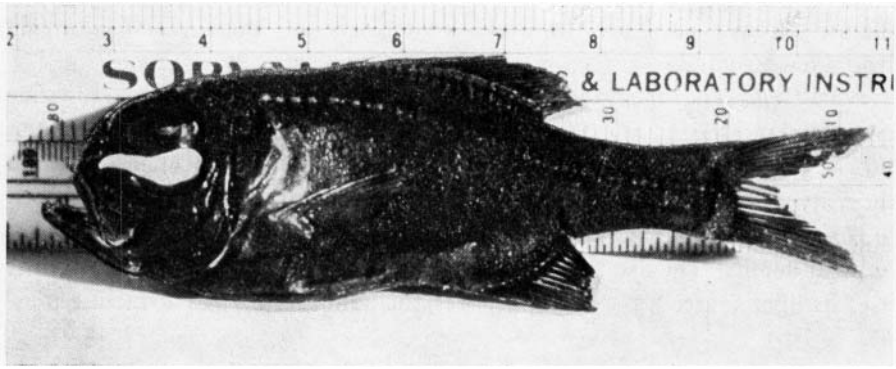

FIG. 1 (Hastings). *Photoblepharon palebratus* from the Red Sea (Marine Biological Laboratory The Hebrew University, Eilat, Israel). The light organs, situated just beneath the eyes and sharing the orbits with the eye, derive their light-emitting ability from symbiotic luminous bacteria which are cultured in the organ. The light can be turned off by the use of a lid. The fish inhabits the coral reef and uses the light in several different specific ways.

from below. Thus, one function of bacterial luminescence involves a symbiosis with a higher organism.

Keynes: What stimulates the dinoflagellates to emit a flash with every wave? Would not the rate of flow of the water relative to the organism be very small at all times?

Hastings: Eckert (1965, *a,b*) has proposed that the difference in inertia between the cell and its flagellae results in shear in reaction to a wave or other shock movement. This evokes generator-like potentials, which can cause flash-inducing action potentials that are conducted around the periphery of the cell.

Hodgkin: But what is the purpose of that flash?

Hastings: Luminescent flashing of this type, in dinoflagellates as well as in other organisms, is believed to serve as a deterrent to predators. Just how is not certain. In the 'burglar alarm' theory for dinoflagellates, the predation causes bioluminescence, thereby revealing the presence of the predators to their own predators. In this way, the grazing on the dinoflagellate population is controlled. However, the idea that the flash directly frightens or diverts the predator is also tenable.

Kornberg: Why do you think this principally occurs in the marine environment?

Hastings: That is the big question. The answer certainly contains both evolutionary and physiological considerations. Briefly, we don't know; nor do I know of any plausible theories or suggestions. Bioluminescence is largely confined to the marine environment, although there are several terrestrial

manifestations. The only freshwater example is the limpet *Latia naritoides* from New Zealand.

Weis-Fogh: The freshwater locality differs enormously from a marine environment; the marine thermocline lies much lower than in fresh water. So, large vertical migrations are possible as well as large changes in light intensity which are absent in the freshwater localities and on land.

Hastings: Yes, but bioluminescence in the ocean is not restricted to organisms at great depths. The fish *Photoblepharon*, dwelling in the coral reef, apparently uses its light source for several purposes, including seeing and attracting prey.

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The purple membrane of *Halobacterium halobium:* a new system for light energy conversion

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Abstract Patches of a distinctly different structure from the rest of the cell membrane occur in *Halobacterium halobium.* The isolated patches are called the purple membrane. It derives its colour from a retinal–protein complex, bacteriorhodopsin, which is the only protein species in this membrane serving a phototransducing function. Light energy is converted by a photochemical cycle going on continuously under illumination and accompanied by a cyclic release and uptake of protons. In the intact cell, this cycle operates as a vectorial process and therefore builds up an electrochemical gradient across the cell membrane conserving part of the absorbed light energy. The cell apparently uses this electrochemical gradient for the synthesis of ATP. Photophosphorylation is shown to be insensitive to cyanide but sensitive to dicyclohexylcarbodiimide (DCCD) and uncouplers. The concentration of ATP and the pH are tightly coupled but can be uncoupled by DCCD. Bacteriorhodopsin, as a light-driven proton pump, can then be studied in the cell as an isolated process. Quantitation of light energy conversion is possible by the indirect method of inhibition of respiration by light and the comparison of the number of absorbed quanta which prevent consumption of one molecule of oxygen.

Photophosphorylation is the most characteristic property of photosynthesizing organisms and occurs in plants and bacteria containing chlorophyll. *Halobacterium halobium* does photophosphorylate but no chlorophyll has been detected in this extreme halophilic bacterium. The photophosphorylation is mediated by a retinal–protein complex, bacteriorhodopsin, which serves a phototransducing function unlike the well-known visual pigments, another class of retinal–protein complexes which serves a photosensing function.

Bacteriorhodopsin is embedded in patch-like areas of the bacterial cell membrane. With growth-limiting aeration these patches occupy about 50% of the total membrane area (Oesterhelt & Stoeckenius 1973). They have been isolated from bacterial cell lysates and termed the purple membrane (Fig. 1). The conversion of light energy absorbed by the chromophore of bacteriorho-

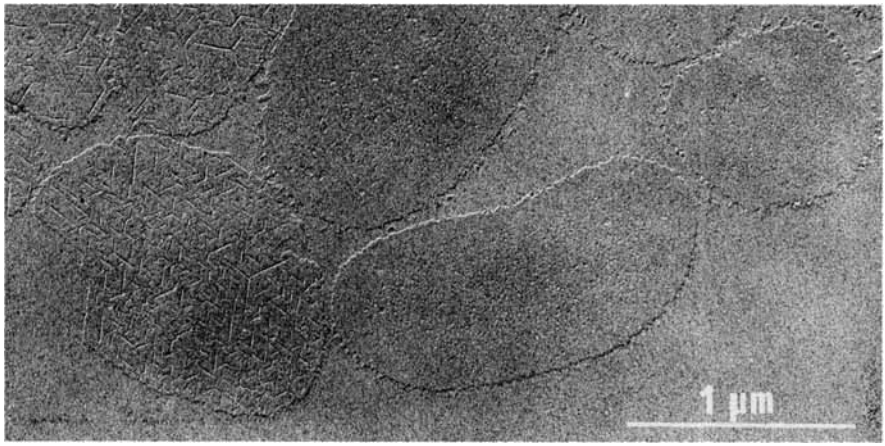

FIG. 1. Electron micrograph (negative print) of purple membranes sprayed on mica and shadowed with platinum–carbon at an angle of 45°. The cracked and the smooth appearance of the sheets show the two different faces of the membranes. (Picture taken by M. Claviez.)

dopsin into free energy of hydrolysis stored in the ATP molecule is now believed to proceed by the following experimentally accessible steps:—

(1) Absorbed light energy drives a photochemical cycle in bacteriorhodopsin accompanied by cyclic release and uptake of protons.

(2) In the intact cell, owing to the orientation of bacteriorhodopsin, step (1) acts as a light-driven proton pump converting light energy into the free energy of an electrochemical gradient.

(3) This energy is used for phosphorylation according to Mitchell's chemiosmotic hypothesis.

(4) Respiration in *H. halobium* is inhibited by light, a fact which allows quantitation of the conversion of light energy by the measurement of the number of absorbed quanta necessary to prevent the consumption of one molecule of oxygen.

In this paper, I shall emphasize the experimental demonstration of photophosphorylation and its relation to light-induced changes in pH.

THE PHOTOCHEMICAL CYCLE IN BACTERIORHODOPSIN

Bacteriorhodopsin is the only protein in the purple membrane which can be easily isolated and studied in aqueous suspensions (Oesterhelt & Stoeckenius 1971, 1974). The chromophore of bacteriorhodopsin absorbs maximally around 560 nm and is called the purple complex. The covalent structure of a

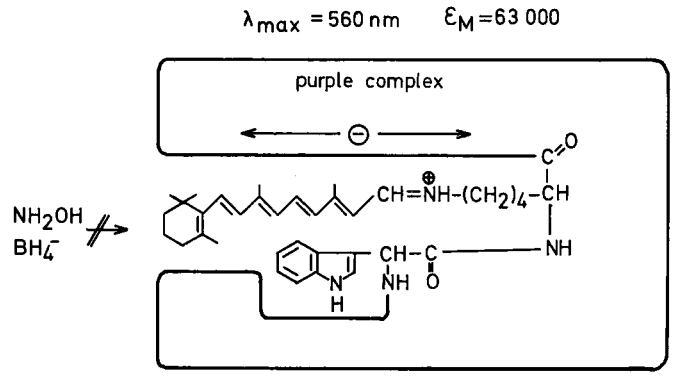

FIG. 2. Binding site of retinal in bacteriorhodopsin. Hydroxylamine and borohydride do not react with the retinal in the purple complex.

protonated retinylidene protein (λ_{max} 440 nm) interacts with the amino acid side-chains, probably the tryptophyl residues, to produce the chromophore with an absorption maximum and chemical reactivity different from a retinylidene protein (Fig. 2). All-*trans*- and 13-*cis*-retinal constitute the chromophore; the two forms of the purple complex differ slightly in absorption maxima and extinction coefficients (Oesterhelt 1971*a*,*b*; Oesterhelt *et al.* 1973).

Light converts the chromophore completely into its all-*trans* form, from which an equilibrium mixture of 13-*cis* and all-*trans* purple complex is slowly formed in the dark, with a half time of about 20 min (Fig. 3). It is not known whether the light-induced conversion of the 13-*cis* purple complex into the all-*trans* purple complex occurs through the 412 nm complex or directly (see Fig. 3). However, only the all-*trans* purple complex seems to be an intermediate in the photochemical cycle in Fig. 3. It is converted by light into the 412 nm complex which spontaneously transforms back into the purple complex within milliseconds. In contrast to the purple complex, the 412 nm form of the chromophore reacts with hydroxylamine to yield retinaloxime and bacterioopsin (Oesterhelt *et al.* 1973). This reaction proved to be a most useful tool in demonstrating the photochemical cycle in intact cells and in the reconstitution of bacteriorhodopsin (Oesterhelt *et al.* 1974; Oesterhelt & Schuhmann 1974). The steady-state concentration of the 412 nm complex ([412]) under continuous illumination and at constant pH is given by equation (1), where [B] is the

$$[412] = [B] / (1 + k_2/k_1 I) \quad (1)$$

bacteriorhodopsin concentration, I the light intensity, and k_1 and k_2 the rate constants (see Fig. 3). Because the existence of more intermediates in the

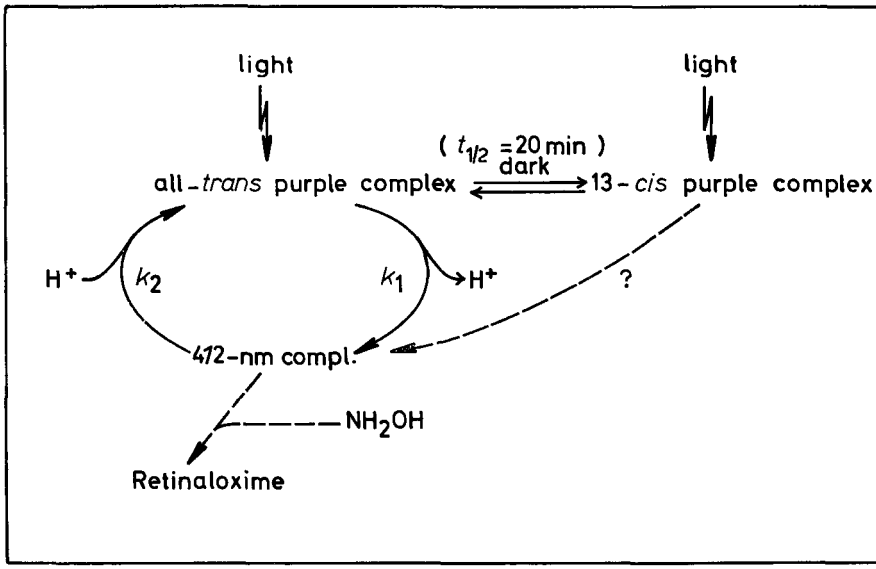

FIG. 3. Photochemical cycle in bacteriorhodopsin.

photochemical cycle cannot be excluded, the rate constants k_1 and k_2 have to be considered as describing the rate-limiting steps leading to the formation of the 412 nm complex and the reformation of the purple complex, respectively.

Even in strong sunlight, the photoequilibrium favours the purple complex so that a purple membrane suspension does not visibly change its colour. We can observe substantial amounts of the 412 nm complex in two ways: by using increased light intensities and by manipulation of the ratio k_2/k_1.

For spectroscopic experiments, the second way has practical advantages. If purple membranes are suspended in 4M-NaCl saturated with diethyl ether, the kinetic constants decrease so that the cycle proceeds over a period of seconds. In addition, the ratio k_1/k_2 is changed so that already-low light intensities will produce the 412 nm complex as the main component in the photoequilibrium. No explanation for this effect of diethyl ether on the rate constants of the cycle can be given, but it is a specific effect not shown by any other organic solvent tested. The salt–ether system allows the separate measurement of the photolytic reaction when the light is turned on and its reversal when the light is turned off. From detailed studies of this system, the following characteristics of the photochemical system most important for its *in vivo* function have been established (Oesterhelt & Hess 1973):—

(1) The 412 nm complex has a broad absorption band with λ_{max} around 415 nm and about half the molar extinction of the purple complex.

(2) The light-dependent formation of the 412 nm complex is independent of temperature; only light absorbed by the purple complex is efficient and the quantum yield is 0.79.

(3) The reformation of the purple complex depends on temperature; the activation energy is 47.7 kJ mol^{-1}.

(4) Formation of the 412 nm complex changes the tryptophyl fluorescence of bacteriorhodopsin and its pK value, i.e. protons are released on illumination and taken up during regeneration of the purple complex.

A second way to influence the photoequilibrium lies in the temperature-dependence of k_2. Lowering the temperature at a given light intensity must influence the photoequilibrium by slowing down the regeneration of the purple complex and, therefore, increasing the steady-state concentration of the 412 nm complex. This formation of the 412 nm complex on illumination to a temperature-determined steady-state as well as its decay in the dark have been demonstrated in intact cells at temperatures below 0 °C (Oesterhelt 1974). The steady-state concentration of the 412 nm complex can be quantitatively related to the temperature dependence of k_2 and we could find no further intermediate with a life time longer than some microseconds spectroscopically (Hess & Oesterhelt 1975). We, therefore, consider the cycle of Fig. 3 as the simple but correct description of the photochemical behaviour of bacteriorhodopsin *in vivo*.

The most important feature of the cycle is the participation of protons, which serve as a link between the photochemical event in bacteriorhodopsin and the cell metabolism. Protons are split off the purple membrane when the chromophore of bacteriorhodopsin is converted by light into its 412 nm complex state and taken up again when the purple complex is regenerated. This cyclic release and uptake of protons was first observed in salt–ether suspensions of the purple membrane (Oesterhelt & Hess 1973), but can also be demonstrated in aqueous suspensions with high light intensities. Also, the release of protons like the formation of the 412 nm complex is independent of temperature, indicating that it is part of the photochemical reaction. Uptake of protons requires about the same energy of activation as the regeneration of the purple complex. In other words, absorbed light energy forces bacteriorhodopsin to change its conformation into a state of higher energy and lower pK value. This state then relaxes in a thermal process to the original conformation. We do not know the exact size of this pK change but nearly one proton is released for every 412 nm complex formed.

In the intact cell, bacteriorhodopsin in the purple membrane is oriented

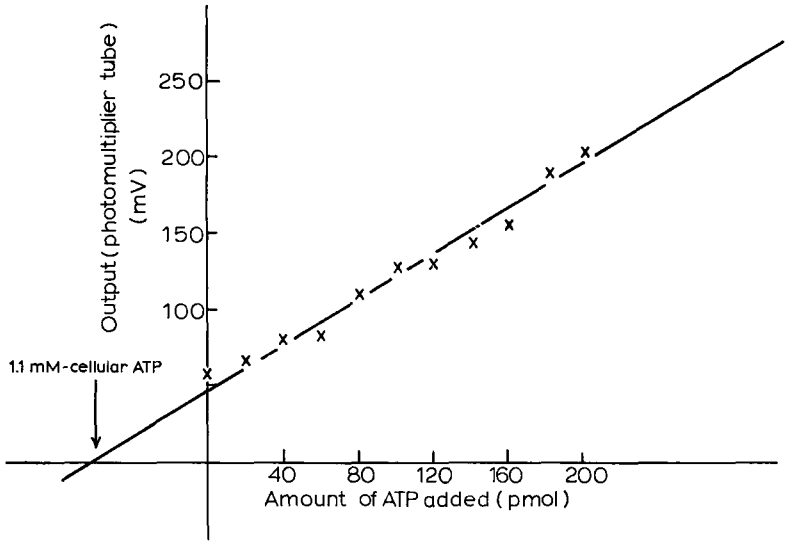

FIG. 4. The concentration of ATP in aerated *H. halobium* cells. Aliquot samples (0.1 ml) of the cell suspension were pipetted into water (5 ml) at 0 °C and 0.5 ml of this solution was mixed with the different amounts of ATP (see abscissa). The bioluminescence was assayed in a SKAN XP 2000. An automatic syringe (Fisons) was used for the addition of buffer (100mM-Tris-HCl and 10mM-$MgSO_4$ pH 7.5) and enzyme (Luciferin–Luciferase, Serva). The absorbance of the cell suspension, 4.0, corresponds to 0.63% internal volume.

asymmetrically, as was shown by the structural work of Blaurock & Stoeckenius (1971) (see also Fig. 1). This asymmetry clearly introduces the possibility that the photochemical cycle is a vectorial process in the intact cell and that protons can be released to one side (i.e. the medium) and taken up from the other side (i.e. cytoplasma). An electrochemical gradient should be created by such a process and could account for photophosphorylation. This proposal was made two years ago, when I started my experiments on the light-induced pH changes in *H. halobium* cell suspensions (Oesterhelt 1972). In the meantime, bacteriorhodopsin has been suggested to act as a light-driven proton pump by *in vivo* as well as *in vitro* results (Oesterhelt & Stoeckenius 1973; Oesterhelt 1974; Racker & Stoeckenius 1974; Kayushin & Skulachev 1974). However, all the observations of light-induced pH changes are so far only qualitative and even at that level they are not always easy to interpret.

PHOTOPHOSPHORYLATION

Cells were resuspended in their physiological medium and 10mM-alanine was added. The absorbance of such suspensions was correlated to internal cell

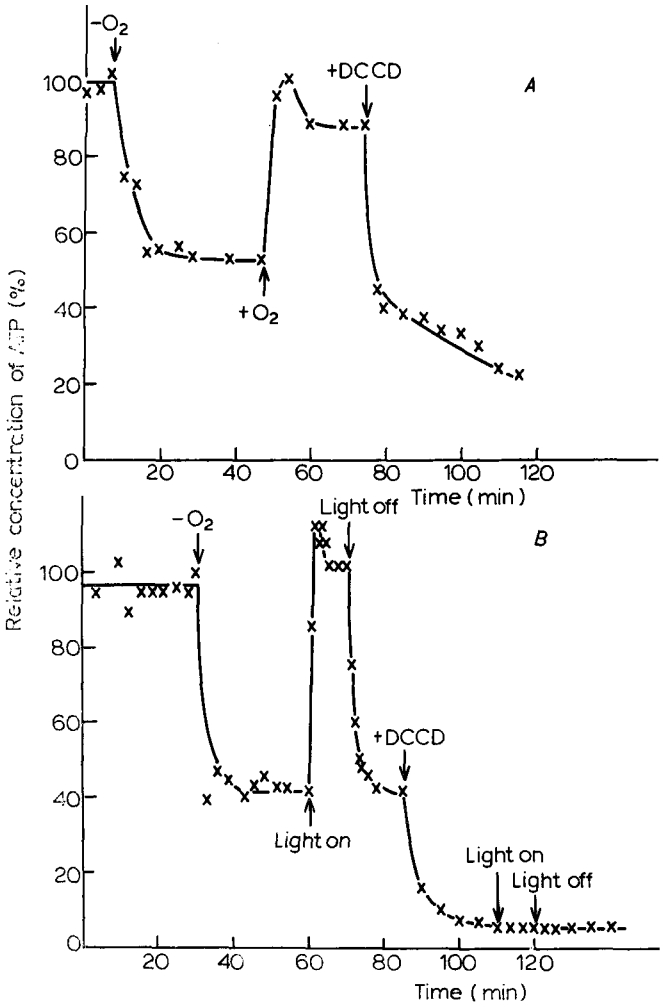

FIG. 5. Oxidative phosphorylation (*A*) and photophosphorylation (*B*) in *H. halobium*. The cell suspensions contained 10mM-alanine and had an absorbance of 8.0 which corresponds to 1.26% internal volume; temperature 25 °C. (ATP was determined as described in Fig. 4.) The aeration was stopped ($-O_2$) by substitution of nitrogen for air and later restarted ($+O_2$). In both *A* and *B* the inhibitor, dicyclohexylcarbodiimide (DCCD) was added to a final concentration of 1 mmol/l.

volume by a calibration curve obtained after microhematocrit centrifugation of the cells. For anaerobic conditions we used closed vessels in which the residual oxygen was quickly consumed or open vessels flushed with nitrogen. The cell suspensions were illuminated with orange light (500–650 nm) selected from

white light by glass filters. The preparation of samples for determination of ATP is convenient with *H. halobium* cells because we can inject aliquot samples into a 50-fold excess of cold water which rapidly lyses the cells and gives a stable solution of ATP. We determined the amount of ATP by the luciferin–luciferase assay (e.g. Bergmeyer 1970). The concentration of ATP in the cells ranged from 1 to 3 mmol/l depending on the cell preparation. A typical determination of the cellular ATP content with added ATP as an internal standard is illustrated in Fig. 4.

The concentration of ATP drops to 30–50% of its original value when the suspension is flushed with nitrogen instead of air (Fig. 5*A*). Apparently, a new steady-state concentration of ATP is established and kept constant by the counterbalance of reactions consuming ATP and the residual synthesis of ATP. The nature of this residual ATP synthesis is not known. When the nitrogen atmosphere is replaced by air, the ATP concentration resumes its initial value after a slight overshoot the significance of which is still unknown. Addition of an inhibitor of the synthesis of ATP dicyclohexylcarbodiimide (DCCD) lowers the concentration of ATP even further than in anaerobic conditions (Fig. 5*A*). When an anaerobic cell suspension is illuminated instead of reaerated (Fig. 5*B*), light replaces oxygen in the phosphorylation. After the light has been turned off the concentration of ATP drops to that seen before illumination whereupon the addition of DCCD almost completely eliminates ATP from the cells. The ATP is lost much faster than in the aerobic conditions of Fig. 5*A*. Once the inhibitor has been added light cannot increase the amount of ATP to a measurable extent. Therefore, we conclude that oxidative phosphorylation as well as photophosphorylation both use a DCCD-sensitive enzyme system, possibly the same one.

Photophosphorylation in *H. halobium* is further characterized by its insensitivity to cyanide and sensitivity to uncouplers. Fig. 6 shows these effects on aerated cell suspensions with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and demonstrates the different sensitivities of oxidative phosphorylation and photophosphorylation to this uncoupler. Whereas oxidative phosphorylation is abolished with 10 μM-hydrazone (Fig. 6*B*), photophosphorylation is still partially active and is only abolished at a concentration of 30 μmol/l (Fig. 6*C*). This higher resistance to an inhibitor can be interpreted in terms of a more efficient proton pump but it could also be due to reaction of the hydrazone with the thiols of the respiratory chain (Kaback *et al.* 1974). The comparatively high concentrations of uncoupler that we used are due to the high titre of the cells in the suspensions necessary for accurate ATP determination. The effect of CCCP depends not only on its concentration but also on the ratio of CCCP to cell mass.

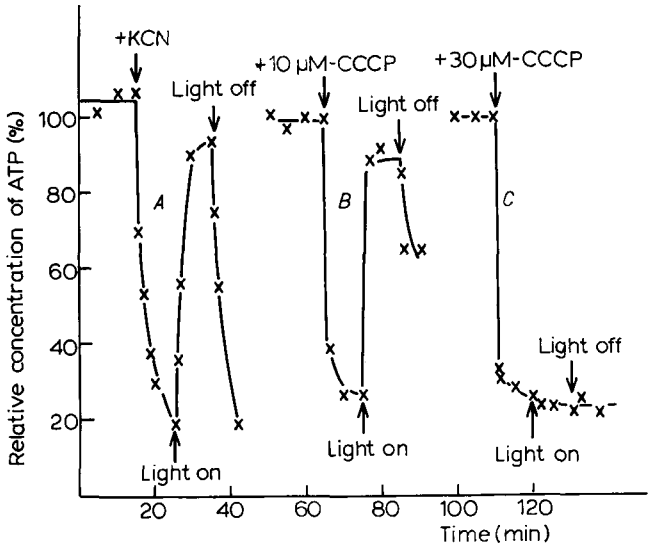

FIG. 6. Influence of cyanide and uncoupler (carbonyl cyanide 3-chlorophenylhydrazone, CCCP) on photophosphorylation. The concentration of ATP was determined as described in Fig. 4: (*A*) KCN was added to a final concentration of 5 mmol/l; (*B*) 10μM-CCCP added; (*C*) 30μM-CCCP added.

LIGHT-INDUCED CHANGES IN pH AND PHOTOPHOSPHORYLATION

Respiring *H. halobium* cells, like other prokaryotic cells and mitochondria, translocate protons to the medium and, according to Mitchell, couple the uptake of protons to phosphorylation in an enzymic process catalysed by the proton-translocating ATPase (West & Mitchell 1974). This leads to a constant pH in the unbuffered medium until oxygen is replaced by nitrogen. The decreasing partial pressure of oxygen apparently stops progressively the synthesis of ATP (see Fig. 5) and consequently an acidification of the medium is seen until oxygen is completely exhausted (Fig. 7). In the dark, the solution outside the cells then remains more acidic than that inside the cells, as indicated when the medium became more alkaline after the addition of CCCP (Fig. 7). This electrochemical gradient does not relax within the duration of the experiments described here.

If, instead of adding an uncoupler, we illuminate the anaerobic cell suspensions, we observe at least three qualitatively different changes in pH, depending on age of the cells and the growth condition used (Fig. 8): (1) the medium becomes more acidic in a process that recalls a dampened oscillation (Fig. 8*A*); (2) the medium becomes more alkaline with an intermediate overshoot in

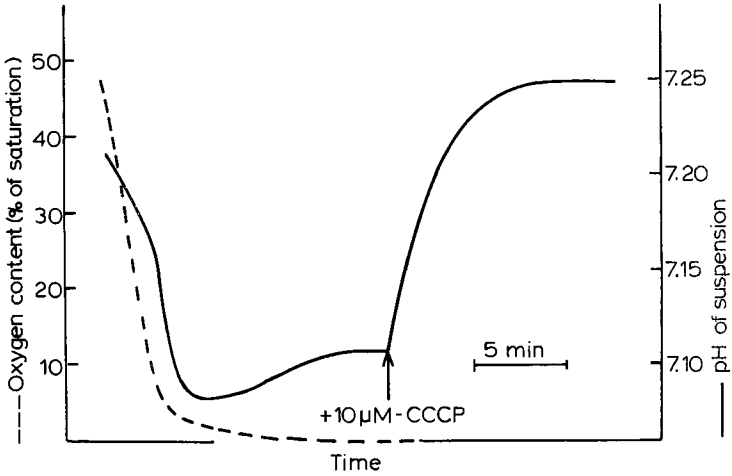

FIG. 7. The change in pH (——) after the oxygen supply (– – – –) to a cell suspension of *H. halobium* has been stopped. The absorbance of the suspension (4.0) corresponds to an internal volume of 0.63%.

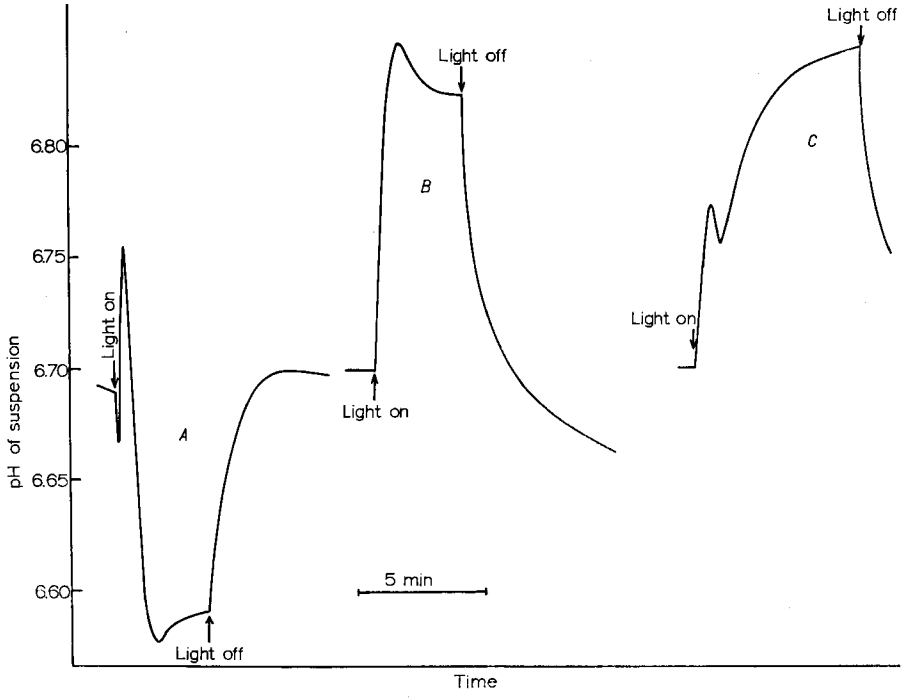

FIG. 8. Light-induced pH changes in *H. halobium: (A)* cells from a fermented culture grown in conditions as described elsewhere (Oesterhelt *et al.* 1973); *(B)* cells from a cell culture on a shaker; *(C)* cells from a cell culture (on a shaker) aerated differently than in *(B)*.

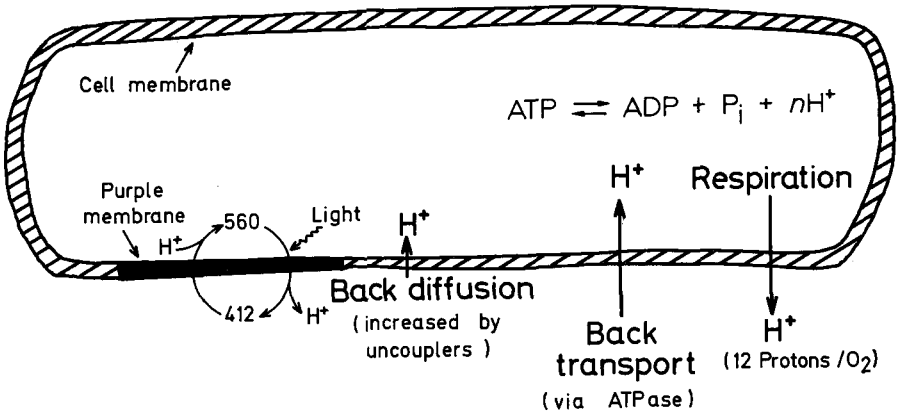

FIG. 9. Scheme for transport of protons in *H. halobium*.

pH (Fig. 8*B*); and (3) the medium becomes more alkaline with an intermediate reverse in sign of the pH trace (Fig. 8*C*).

Unfortunately, we cannot correlate quantitatively the light-induced changes in pH of a cell suspension with the bacteriorhodopsin content and the capacity of the cell to synthesize ATP. However, we can and must find an explanation for the medium becoming acidic or basic upon illumination of different cell preparations, since we cannot assume that bacteriorhodopsin acts as a light-driven proton pump in one case and as a hydroxy ion pump in other cases. First, we have to consider the processes coupling pH with ATP concentration in the cells in anaerobic conditions (Fig. 9):—

(1) Light drives the ejection of protons from the cell in an electrogenic process.

(2) The synthesis of ATP translocates protons back into the cells and is accompanied by a net disappearance of protons according to the pH dependence of its formation (West & Mitchell 1974).

(3) Hydrolysis of ATP produces the exactly reverse changes in pH to those caused by its synthesis.

(4) Passive diffusion of protons in either direction tends to equalize differences in pH between the inside and outside of the cell and is enhanced by uncouplers.

Stopping the oxygen supply leaves the cell with an excess of protons externally which can be used for ATP synthesis. Light generates a membrane potential $\Delta\psi$ necessary to satisfy equation (2) where ΔG is the free energy of hydrolysis for

$$\Delta G = -RT\ln [H^+_{out}/[H^+_{in}] - nF\Delta\psi \qquad (2)$$

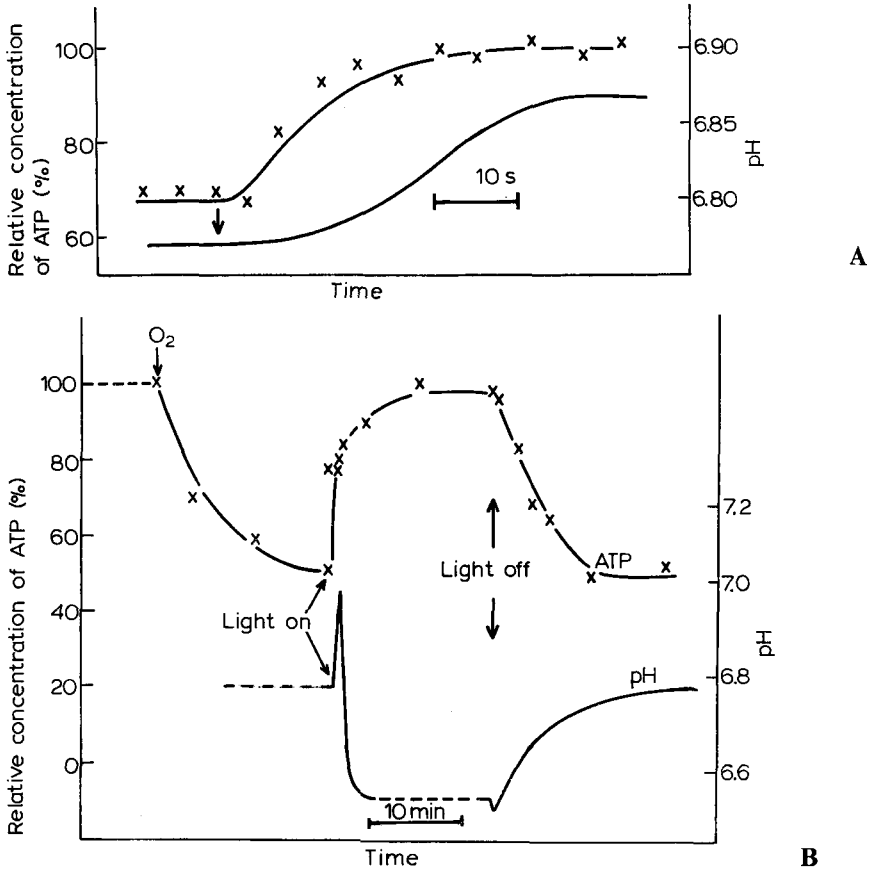

FIG. 10. Changes in photophosphorylation (measured as the relative concentration of ATP in %: ×) and in pH (——). *(A)* Light-induced synthesis of ATP during the transient increase of pH; *(B)* overall changes on illumination and in the dark.

ATP in the conditions in the cell. In a very short time (less than one millisecond), this membrane potential is created by bacteriorhodopsin as an electrogenic pump and ATP synthesis starts with back translocation of protons into the cell causing the medium to become alkaline until the relative concentration of ATP reaches 100%. Then, proton uptake is limited by metabolic reactions which consume ATP and bacteriorhodopsin acidifies the medium until it reaches an equilibrium with passive back diffusion of protons and proton translocation for ATP synthesis. We do not yet know whether potassium is the counter-ion taken up during this electroneutral acidification, but it can be considered as a suitable candidate since it is taken up by the cells to high concentrations

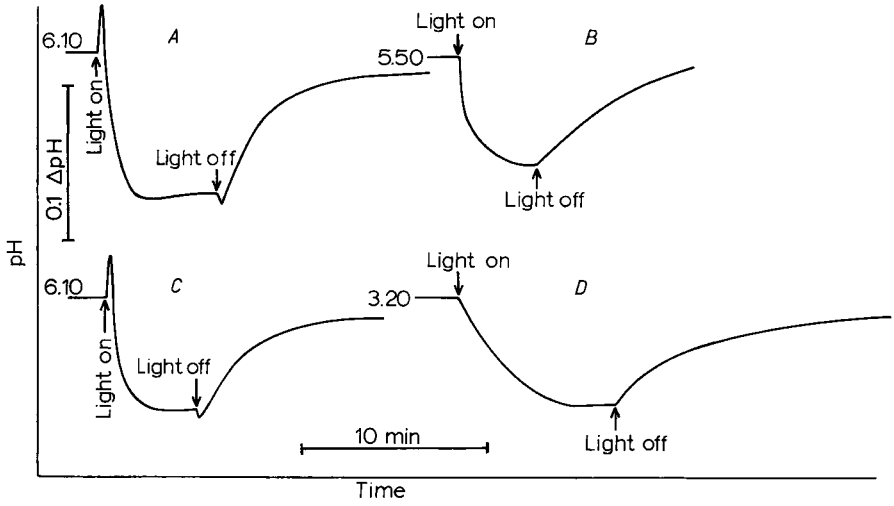

FIG. 11. Light-induced changes of pH at different acidities of the medium: *(A)* pH 6.10; *(B)* pH 5.5; *(C)* pH 6.10; *(D)* pH 3.2. All four measurements were made with the same sample.

(Ginzburg *et al.* 1971; Larsen 1967). Turning off the light restores the initial pH level.

This proposed scheme could explain the pH trace in Fig. 8*A*, provided ATP is synthesized as fast as the pH overshoots (see Fig. 10*A*). No matter how the pH changes on illumination, ATP is synthesized in any cell preparation during the initial pH change, kept constant at 100% relative concentration as long as the suspension is illuminated and decreases as soon as light is turned off (Fig. 10*B*).

The overshoot in pH is thus caused by proton translocation and the stoichiometric consumption of protons during the synthesis of ATP; it should depend on the pH of the medium. Fig. 11 shows the course of the change in pH in a cell suspension with the typical overshoot at pH 6.10 (Fig. 11*A*) and its reversible disappearance when the medium is acidified to pH 5.5 (Fig. 11*B*) and then readjusted to pH 6.10 (Fig. 11*C*). The overshoot disappears between 5.7 and 5.4 and depends, apparently, on the time of equilibration between the internal and external media. We can calculate that at about pH 5.5 the net consumption of protons by ATP synthesis becomes zero (pK_a values are taken from Nishimura *et al.* 1962). Photophosphorylation is still observed at pH 5.5, but at a reduced rate. This observation may explain why immediate acidification follows illumination. Bacteriorhodopsin, as the acidifying proton pump, overcomes the inwardly directed proton translocation coupled to ATP synthesis.

Photophosphorylation further declines with decreasing pH and is abolished at pH 3, in contrast to the light-driven proton pumping which increasingly acidifies the medium. Titration experiments indicate that at pH 3.2 about 1000 protons per molecule of bacteriorhodopsin present in the cell suspension are ejected at a given light intensity until the influx of protons by passive diffusion equals the efflux by pump action (Fig. 11*D*).

Photophosphorylation in *H. halobium* is unaffected by substances inhibiting cyclic or non-cyclic electron flow in photosynthesizing organisms (Danon & Stoeckenius 1974). This fact, together with the insensitivity of photophosphorylation to cyanide (Fig. 6*A*), makes redox reactions unlikely accompaniments to the photochemical cycle of bacteriorhodopsin. As mentioned before, light can create an electric field across the cell membrane through the action of bacteriorhodopsin as an electrogenic pump. *In vitro* studies on bacteriorhodopsin containing proteoliposomes have given some evidence in favour of this idea (Kayushin & Skulachev 1974; Drachev *et al.* 1974*a*,*b*). The authors claimed that the electromotive force of bacteriorhodopsin is at least 300 mV. From the kinetic data of the photochemical cycle and the bacteriorhodopsin concentration in the cell in standard conditions of growth one can estimate that a membrane potential of 300 mV can be created within a millisecond and that it involves only a small net proton translocation. Such a field makes a large pH gradient unnecessary as a prerequisite of ATP synthesis and explains why synthesis proceeds, even when the medium becomes alkaline (Fig. 8*B*). Then the efficiency of the proton pump in bacteriorhodopsin is not large enough to overcome the inward translocation by ATP synthesis, but creates the field necessary for the synthesis. It is more difficult to interpret the pH trace in Fig. 8*C*, but tentatively one can assume that as illumination starts ATP synthesis the ATP-consuming reactions, which were partially shut off once the oxygen was exhausted, are reactivated so that ATP synthesis requires increasing amounts of protons.

Summarizing our interpretation of the different pH traces in Fig. 8, we can say that light always immediately starts ATP synthesis accompanied by proton uptake of the cells at pH values greater than 5.5. After ATP has reached a relative concentration of 100%, the pH stabilizes at a more acidic level in the case of an efficient light-driven proton pump or at a more alkaline level in the case of a less efficient pump. In all cases, the rate of ATP synthesis needed to keep the concentration at 100% and the proton pump activity will determine the steady-state level of the pH.

If this qualitative interpretation is correct, we should be able to demonstrate the imbalance introduced by shutting off one of the two components, similar to the experiment of Fig. 11*D*. The easiest way to do this is the inhibition of

the synthesis of ATP by DCCD (Fig. 12). Together with the capacity for synthesizing ATP, the overshoot in pH is abolished and acidification is greatly enhanced. This result is independent of the type of light-induced change in pH shown in Fig. 8 and confirms our assumption that protons ejected by the proton pump are used for ATP synthesis. When the ATP synthesis is shut off, the pump is left working alone; a more acidic steady-state results where pump action is counterbalanced by passive back diffusion. This is exemplified in Fig. 12; after addition of DCCD and illumination this steady-state pH is raised by the addition of increasing amounts of CCCP until the pump under the given circumstances is made completely inefficient by a 'leaky' cell membrane.

LIGHT INHIBITION OF RESPIRATION

Measurement of the quantum requirement in photophosphorylation would be the most direct way for quantitation of light energy conversion in *H. halobium*, but since our methods are not yet sufficiently improved for this task we chose light inhibition of respiration as a basis for indirect quantitation of light energy conversion (Oesterhelt & Krippahl 1973). Both systems, bacteriorhodopsin (through light energy) and the respiratory chain (through a redox reaction), provide free chemical energy and must, therefore, compete in the cell with each other. For what do they compete? The three possible prizes are: ADP, protons inside the cell and membrane potential. I should point out again that competition for an electron carrier common to both systems appears most unlikely since there is no indication of a redox chain in the conversion of light energy by the purple membrane (Oesterhelt & Stoeckenius 1973; Danon & Stoeckenius 1974). Competition for ADP also seems unlikely since the proton translocating ATPase system does not distinguish between protons coming from bacteriorhodopsin and protons coming from the respiratory chain. Therefore, competition through the membrane potential seems to be the most likely explanation for light inhibition of respiration. It would be understandable in terms of a counter-voltage (produced by light absorbed in bacteriorhodopsin) applied to the membrane potential created by respiration.

Light inhibition of respiration can be conveniently measured in a Warburg respirometer in which light is projected onto the bottom of one of two vessels containing identical cell suspensions. Up to 30% inhibition is found depending strictly on oxygen saturation of the cells (i.e. only maximally respiring cells show this light inhibition), thereby indicating that the concentration of ATP has to be 100% in order to couple the two systems for competition. The effect needs the light intensity to reach a level of saturation at high light intensities,

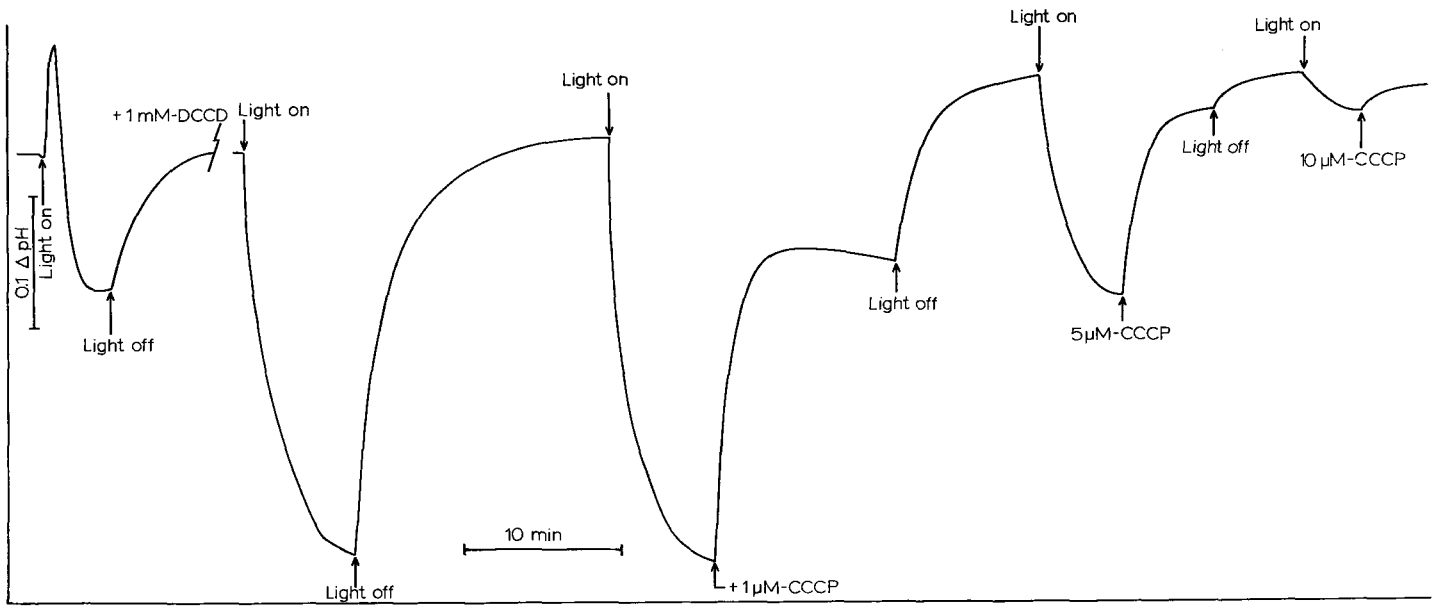

FIG. 12. Uncoupling of the light-induced changes in pH from ATP synthesis by DCCD. The proton-pumping bacteriorhodopsin is made ineffective afterwards by addition of increasing amounts of CCCP.

where the speed of the proton-pumping bacteriorhodopsin reaches its maximum and becomes independent of light intensity. The absorption of the shaking turbid cell suspensions can be measured with an integrating sphere as described by Warburg & Krippahl (1954). The results so far obtained are (Oesterhelt & Krippahl 1973; Krippahl & Oesterhelt 1975):—

(1) Light inhibition of respiration is not due to a general inhibition of metabolism since the cells grow even faster in light than in the dark, but at a reduced rate of oxygen consumption.

(2) Not only reduced oxygen consumption but also reduced consumption of specific substrates (e.g. succinic acid) can be demonstrated.

(3) The action spectrum of light inhibition is identical with the absorption band of the purple complex in bacteriorhodopsin.

(4) At least 24 quanta have to be absorbed to prevent the consumption of one molecule of oxygen. Since no more than one proton can be translocated in one complete photochemical cycle (quantum yield 0.79) and 12 protons are translocated per molecule of oxygen reduced (Scholes & Mitchell 1970; Hinkle & Horstmann 1971) the quantum yield of the light energy converting system will be maximally 0.5, which is a remarkable high value.

CONCLUSION

The conversion of light energy in *H. halobium* requires two proton-translocating systems: the proton-pumping bacteriorhodopsin and an H^+-ATPase. Apparently, no electron flow accompanies the photochemical cycle in bacteriorhodopsin and the action of this molecule can be studied separately from the ATPase system in cells as well as in the isolated purple membrane. This system, therefore, is simpler and its detailed way of function might be easier understood than that of most other energy-transducing processes in biological membranes. Photophosphorylation in *H. halobium* is apparently driven by the electrochemical gradient created by the electrogenic proton pump, bacteriorhodopsin. The membrane potential plays an important role as indicated by ATP synthesis in conditions where the change in pH is still small and ATP hydrolysis immediately after light has been turned off.

The light energy absorbed by bacteriorhodopsin produces a conformational change accompanied by a change in pK. The energy stored in the conformation of the 412 nm complex is used to complete the photochemical cycle and the pK change is used to create the electrochemical gradient. Clearly, energy conservation is possible by changes in conformation as well as by the electrochemical gradient and both are intimately linked. The chances are good that we shall be able in the future to describe quantitatively changes in pH, con-

centration of ATP and competition of light and respiration in *H. halobium*. The fundamental problem, however, even then remains unsolved: what are the molecular processes affecting proton translocation? Bacteriorhodopsin in the purple membrane is well suited for the study of this problem so that we may understand in the future vectorial proton movement, at least in this case.

Beside light energy conversion, which can be called chlorophyll-independent photosynthesis, the purple membrane system fascinates by its other aspects: regulation of its biosynthesis by light and oxygen; the structure, function and evolution of retinal–protein complexes and the structure of the biological membrane.

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Discussion

Huxley: How is the energy stored in the 412 complex?

Oesterhelt: It is stored in the conformation of the bacteriorhodopsin molecule, which differs from the purple complex in the 412 nm complex state. Since the latter spontaneously reconverts into the purple complex, the free energy change of that step must have been put into the system by absorbed light energy. However, we do not know yet the size of this free energy change.

Keynes: The rhodopsin molecule is free to rotate with a relaxation time of the order of microseconds in rods (Cone 1972). Similar experiments on this protein reveal much longer relaxation times (Razi Naqvi *et al.* 1973).

Oesterhelt: Cone (unpublished results, 1973) and Razi Naqvi *et al.* (1973) demonstrated that, in the purple membrane, bacteriorhodopsin does not rotate with a measurable relaxation time.

Keynes: In rhodopsin, the protein behaves as if it is floating in a sea of liquid (see as well p. 187), whereas in your case the structure must be more rigid.

Oesterhelt: Yes, X-ray analysis and electron microscopy reveal a hexagonal crystalline array of the protein in the membrane (Blaurock & Stoeckenius 1971).

Huxley: What is the direction of the absorbing dipole?

Oesterhelt: The dipole lies in the plane of the membrane (Blaurock & Stoeckenius 1971). Also, rhodopsin cannot be a rotational carrier because it rotates in the plane of the membrane not through it.

Hagins: How thick are the patches?

Oesterhelt: X-ray diffraction shows them to be 4.8 nm thick (Blaurock & Stoeckenius 1971).

Huxley: What was the mechanism of ATP synthesis in the first stage?

Oesterhelt: I cannot explain the mechanism of ATP synthesis, but I can provide the formalism to explain why the synthesis can start immediately after the light is turned on. Light—through the proton-pumping bacteriorhodopsin—produces a membrane potential which in turn increases the rate of ATP synthesis.

Huxley: Is there any evidence for this change in membrane potential or, assuming the Mitchell hypothesis, are you calculating membrane potential?

Oesterhelt: Drachev *et al.* (1974) measured an electromotive force of about 300 mV for bacteriorhodopsin incorporated into liposomes (see p. 160). From our measurement of the kinetic parameters of the photochemical cycles we can calculate the speed of the proton pump in our cell suspensions and the time necessary to create a membrane potential of 300 mV in an electrogenic process. The time is less than a millisecond, i.e. more than 1000-fold faster than the observed ATP synthesis.

Roseman: So, the first response is a change in membrane potential that is effectively produced by the proton pump of the cycle.

Oesterhelt: This is what we assume, but we have not yet observed the membrane potential experimentally in intact cells.

Roseman: At the same time as this proton-mediated change on the membrane that allows the inward flow of protons for the generation of ATP, is it not true that a pH gradient is building up inside? This, surely, would tend to repel the protons.

Oesterhelt: Not necessarily, because the pH of the medium during the increase in ATP concentration does not reach the value for equilibration

between the inside and the outside of the cell (see Fig. 7). In addition, a new disappearance of protons due to the proton consuming synthesis of ATP inside the cell occurs:

$$nH^+ + ADP + P_i \rightarrow ATP$$

n is close to 1 at pH values above 7 (see Nishimura *et al.* 1962).

Roseman: If you can induce the proton flux by light at low temperature both *in vivo* and *in vitro*, should you observe some effect on processes in the membrane? Do you see the overshoot, for example, at low temperatures?

Oesterhelt: In the low temperature experiments, no proton flux was measured but spectroscopic changes of bacteriorhodopsin were. As yet, we have not used any indicators to follow pH changes at these temperatures.

Hess: I have observed that the light reaction of the purple membrane is fully inhibited at about –80 °C and the dark reaction is about –40 °C, thereby allowing temperature jump experiments for the observation of the dark reaction in the range of –20 to –30 °C. In the latter range, the pH kinetics can be followed qualitatively with a pH electrode. However, quantitative data and transient-state kinetics have not been obtained yet because of the poor efficiency of the pH electrode in the solvent conditions used.

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Ionic aspects of excitation in rod outer segments

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Abstract The current status of the problem of ionic mechanisms underlying excitation of vertebrate photoreceptors is reviewed. Evidence is presented that the ionic dark current of retinal rods is sustained by the action of a ouabain-sensitive Na–K exchange pump driven by oxidative metabolism. The photoreceptors are depleted of K when the pump is stopped by ouabain. Considerations of cell membrane topology, kinetics of the light response, and signal amplification indicate that the light-induced suppression of the ionic dark current is mediated by a diffusible internal chemical transmitter substance. The desensitizing effect of low Ca^{2+} Ringer's on the light responses of vertebrate rods, along with other indirect evidence, suggests that the transmitter substance may be calcium ions released from the internal membranous disks of the outer segments.

The ionic hypothesis of excitation and conduction in excitable cells (Hodgkin & Huxley 1952; Hodgkin 1964) has been so successful in accounting for nerve action that it is now difficult to think of a drawing of a nerve cell without arrows indicating ionic fluxes, little wheels denoting active transport mechanisms or an electrical circuit diagram of some sort. Partly out of a sense of tidiness and partly in hopes of turning up new principles (or at least new variations on old principles), many scientists have extended the pioneering work of Hodgkin & Huxley to cells large enough for good intracellular recordings with at least three or four electrodes and having enough cytoplasm for chemical analysis. In principle, it is easy to describe the electrical properties of a cell; all we need to know is the cytoplasmic concentration and electrochemical activity of all ionic species to which the plasma membrane is passively permeable, the permeabilities and one-way isotopic fluxes themselves, perhaps as functions of membrane potential, external and internal fluid composition, the membrane capacitance (including its non-linearities) and the fluxes of all ions actively transported through the plasma membrane. If the cell is differentiated into

special regions, then each one must be described separately. The provision of this kind of detailed information about a small neuron seems almost as remote an objective as is the construction of an exact quantum-mechanical description of a molecule containing a hundred atoms.

Nevertheless, we ought to know how vertebrate photoreceptors behave as nerve cells because their sensory function is important and because they stand as the prime example of conversion of a stoichiometric stimulus into a continuous neural response. We shall describe some experiments designed to elucidate their ionic mechanisms. Because the cells are small and their activity is partly mixed with that of other retinal neurons, many of our conclusions are tentative and, at best, only plausible.

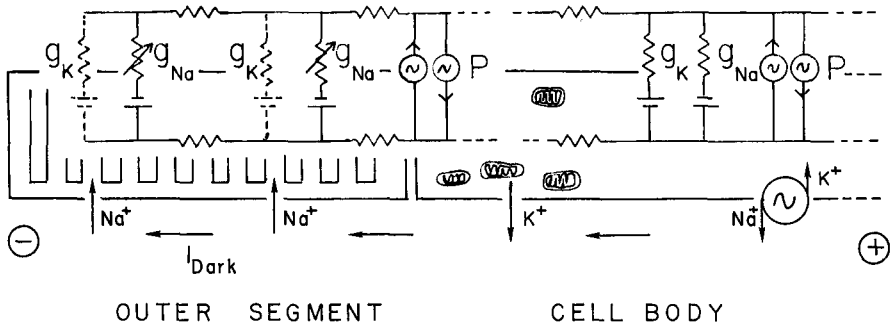

FIG. 1. Schematic diagram of a vertebrate photoreceptor and its equivalent electrical and ionic circuits.

ELECTRICAL PROPERTIES OF VERTEBRATE PHOTORECEPTORS

A retinal rod or cone is usually thought of as a specialized neuron with an electrical equivalent circuit similar to that of Fig. 1. In darkness, a steady electric current, the 'dark current', enters the plasma membrane covering the light-absorbing outer segment, flows axially through the cytoplasm, re-emerges through the surface membrane of the inner segment, nuclear region and synapse and returns to the outer segment in the intercellular space (Penn & Hagins 1969; Hagins *et al.* 1970; Zuckerman 1973; Ernst *et al.* 1974). The flow of the dark current creates a steady potential gradient in the interstitial space. Although initially unexpected by retinal physiologists, the dark current is analogous to the currents maintained by epithelial cells like those of frog skin.

Membrane potentials recorded from such cells with intracellular microelectrodes show that the membranes are somewhat depolarized in darkness but the membrane potentials increase in light (Tomita 1970; Baylor & Fuortes

1970; Baylor & Hodgkin 1973; Brown & Pinto 1974). Similarly, light suppresses the dark current with the same time course and to about the same degree as the membrane potential is increased towards its maximally hyperpolarized value. It was immediately recognized that the two electrical effects were probably aspects of the same fundamental response, a light-induced change in the properties of the receptor membranes (Tomita 1970). Subsequently, much effort has been directed towards answering three questions. (1) What ions carry the dark current? (2) By what electrical effect does light suppress it? (3) How do signals from the photopigment molecules reach the plasma membrane of rods and cones?

IONIC BASIS OF THE DARK CURRENT

Because vertebrate rods and cones look much like other neurons, one would suspect that their ionic batteries are charged by accumulation of K^+ and extrusion of Na^+ by Na–K transport processes at the plasma membrane. However, it has been difficult to show that the ionic composition of vertebrate photoreceptors is like that of ordinary brain tissue because such a large fraction of the retina consists of non-receptor cells. Isolated rod outer segments have not yielded clear-cut ionic compositions and isotopic fluxes (Cavaggioni *et al.* 1973; Arden & Ernst 1971), partly for reasons we shall consider later. So, we have made a direct attempt to estimate the ionic composition of the layers of fresh frozen, dark-adapted, frog retina by the electron microprobe technique. This method has long been under development in many laboratories but is beginning to show useful semi-quantitative results on quick-frozen tissues that have not been dried or infiltrated with embedding substances.

Fig. 2 shows a typical set of scans of a dark-adapted retina. The entire retina, including the layer of rod outer segments, has relatively high concentrations of K^+ and sulphur and little chlorine or calcium. Numerous aggregates of calcium can be seen: these are dust particles from an untidy laboratory and not retinal constituents. Qualitatively, the scans are what would be expected of any neural tissue containing sulphur-bearing proteins and cells that accumulate K^+, extrude Na^+ and allow Cl^- to diffuse passively across their plasma membranes. Although one can count photon densities, the value of such area scans is mainly qualitative. More accurate results require line-profile scans across the retinal layers. With our present technique, adequate photon counting rates ($>$ 14 counts/s) require electron beam currents of more than 20 nA. This accelerates sublimation of the ice and causes grooves in the surfaces of the specimens so that repeated scans of the same area cause 10–20% loss of signal with each successive scan. Nevertheless, relatively stable and reproducible

S
r.o.s
FILTER
ICE
RETINA
Ca
Cl
K

element profiles can be obtained if one is satisfied with 10–20% analytical precision at 5 μm spatial resolution.

Fig. 3 shows K, Cl, and Fe line profile scans through two frozen retinas. Each profile is the average of two taken along parallel tracks about 50 μm apart. Despite the irregularities in the tracings, due in part to etching by the electron beam and irregular contour of the cleaved surface of the specimen, the relatively high K and low Cl concentrations in the receptor layer are visible. We intended to estimate from the iron scan the interstitial space in the outer segment layer by its infiltration with ferritin (of 10% iron content). However, the ferritin penetrated incompletely and we computed the estimated compositions of the outer segments instead by correcting for the presence of about 35% interstitial space estimated from light micrographs. To establish that the composition of the photoreceptors was influenced by ion transport pumps, we studied the effect of ouabain on both the dark voltage gradient and elemental composition of frog retina.

Treatment of the live frog retina with 50μM-ouabain in Ringer's causes the

← FIG. 2. Analysis of the elemental composition of a dark-adapted and quick-frozen retina from *R. pipiens* by the electron microprobe technique (see Heinrich 1968). Upper right: secondary electron scan of a retina–filter combination fractured perpendicular to the plane of the retina (see sketch, centre right; filter is 180 μm thick—uncorrected for thermal contraction of the ice at −150 °C). The retina was dissected in darkness (see Hagins *et al.* 1970), attached by suction to a Millipore (type HA) filter disk and immersed in darkness for 10 min in a frog Ringer's (115mM-Na^+, 2.7mM-K^+, 1.36mM-Ca^{2+}, 1.25mM-Cl^-, 10mM-Tris, 100μM-Mg^{2+}, 11mM-glucose, and 1% ferritin). The retina was frozen by quick compression between two polished copper blocks at −196 °C, then fractured and mounted in a holder attached to the cold stage of a scanning electron microscope (Etec) fitted with a wavelength-dispersive X-ray spectrometer. No conductive coating was applied to the fractured surface until after the elemental scans were finished. All specimens were scanned at between −145 and −150 °C with a 10 kV electron beam and a specimen current of 20–50 nA. Repeated area scans could be made for at least 18 h without sublimation of more than 2–3 μm of the ice surface. The sublimation rate increased to about 0.1 μm s^{-1} under a static 40 nA electron beam. Preliminary horizonal line profile-scans through the centre of the preparation etched two grooves visible in the secondary electron scan.

Element scans: Each scan required 36 min to produce the density of X-ray photons shown. Because of the large area covered, not all parts of it produced X-rays equally well accepted by the spectrometer's exit slit. The orientation of the slit image is shown in the sulphur scan (S); the short cross-bar indicates the half-width of the spectrometer slit acceptance zone. At distances greater than three half-widths from the diagonal slit image, the X-ray count density is essentially that of the X-ray continuum near the selected K α line. The scans (all with the pentaerythrytol crystal of the spectrometer set on the appropriate elemental K α line) show the high K and S and low Cl content of the retinal layers. Separate scans across the K α emission lines of the elements in frozen solutions indicated that the concentrations of elements needed to yield counting rates that were twice that of the X-ray continuum from ice were 1–3 mmol/l for S, Ca, Cl and K. The depth of the specimen analysed is about 4 μm. The sulphur scan shows a marked shadowing of the X-rays emitted from the bottom of the groove etched by the preliminary line-profile scans.

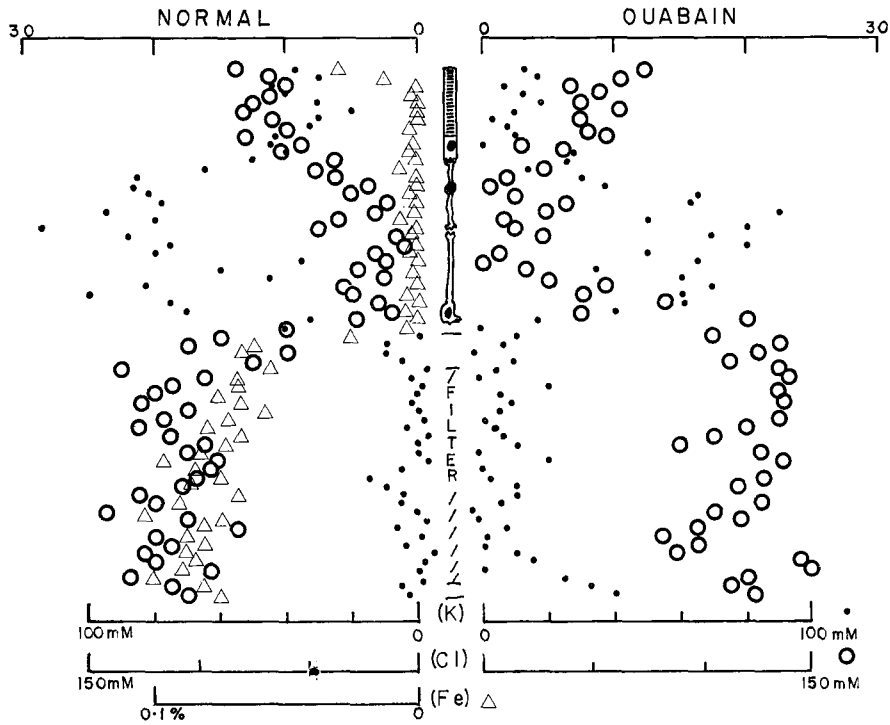

FIG. 3. Line-profile scans (top scale in counts/s at 40 nA specimen current) across two frozen retina–filter preparations (as in Fig. 2). Each scan took 4 min to traverse the retina and filter. Left: K(●), Cl(○) and Fe(△) scans across a dark-adapted control retina. Concentration scales (bottom) are computed from counting rates of frozen solution standards of known composition on the assumption that X-ray count rates are proportional to weight fractions of each element when corrected for counts from the X-ray continuum. The elaborate corrections needed for analysis of alloys and dense minerals (Heinrich 1968) were not made since the sample always contained more than 80% H_2O and otherwise contained elements of low atomic number. Right: K and Cl scans across a retina incubated at 25 °C for 10 min in the frog Ringer's of Fig. 2 (without ferritin) containing 50μM-ouabain. The K^+ content of the receptor layer of this retina was much less than that of the control, while its Cl content was about the same. Evidently loss of K was not accompanied by a significant gain of Cl^-. This result is consistent with the observation (Arden & Ernst 1971) that the Na^+ content of rods is increased by ouabain treatment.

standing voltage gradient in the rod layer to diminish steadily with a half-time of about 4 min (Zuckerman 1973) (Fig. 4). This effect, which takes only about 1 min in the smaller rods of rats (Yoshikami & Hagins 1973), suggests that retinal rods, like most cells, depend on Na–K exchange transport pumps in their plasma membranes to sustain their electrical activity. However, we have not so far observed a change in the shape of the voltage profile immediately on

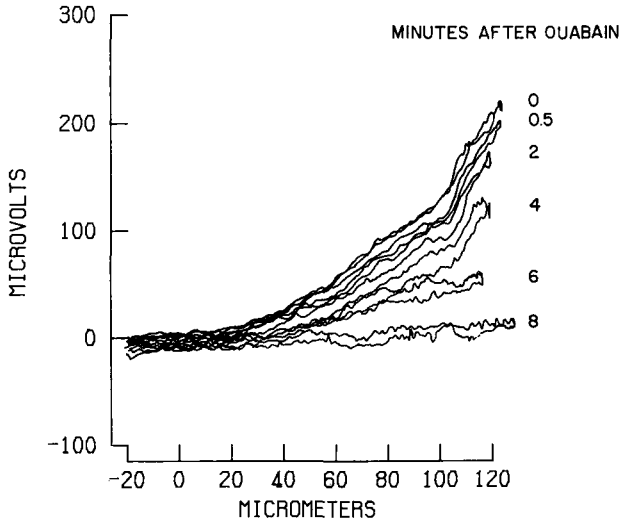

FIG. 4. Extracellular voltage plotted against electrode depth in receptor layer of *R. pipiens* retina in a flow chamber before and during exposure to 50μM-ouabain Ringer. Rod outer segments extend from 0 to 50 μm; rod synapses at 85 μm. Recordings made with 1 MΩ Ringer-filled pipettes; fixed pipette at −10 μm, moving pipette attached to a micromanipulator whose position was coupled to a motion transducer that generated the abscissa of the graph. Repeated cycles of insertion and withdrawal of the moving electrode shown before ($t = 0$) and at intervals after application of ouabain. The monotonic dark-voltage gradient collapsed smoothly as the dark current slowly disappeared. No fast change in shape of the gradient attributable to suppression of an electrogenic Na pump in the rods was seen. Ionic analysis of a similarly treated retina is shown in Fig. 3.

application of ouabain Ringer's such as was ascribed by Zuckerman (1973) to inhibition of a localized electrogenic Na pump in the inner segments. In all our experiments, the dark-voltage gradient smoothly collapsed as the dark current disappeared.

Elemental scans for K and Cl through such a retina exposed to ouabain for 10 min in darkness are shown in Fig. 3. The Cl content of the rod layer did not differ significantly from that of the control retina, but the K content was greatly diminished. Table 1 shows estimated K and Cl contents for retinal rods derived from data like those in Fig. 3. If the chloride is entirely in the form of dissociated ions, E_{Cl} for the rod membranes is about −35 mV, a value not far from the resting membrane potential measured in single visual cells with microelectrodes (Baylor & Hodgkin 1973; Baylor & Fuortes 1970).

TABLE 1

Estimated ionic contents of rods from *R. pipiens* derived from electron probe element profiles through two frozen retinas. Errors are estimates based on counting statistics and measured instabilities of counting rates due to etching of the ice by the electron beam.

Specimen	*[K]/mmol l^{-1}*	*[Cl]/mmol l^{-1}*	
Ringer's	2.7	125	
Normal retina			
Outer segment cytoplasm[a]	25 ± 7	30 ± 10	1
Nuclear layer[b]	50 ± 5	20 ± 10	2
Retina treated with ouabain			
Outer segment cytoplasm[a]	< 5	20 ± 10	1
Nuclear layer[b]	10 ± 5	20 ± 10	2

[a] Average K α-photon counting rate (corrected for X-ray continuum) for 50 μm wide region extending from outer segment tips to junctions of inner and outer segments. The extracellular space was estimated at 35%. Values are uncorrected for 40% solids in outer segments.
[b] Calculated as in *a* for a 25 μm wide zone extending proximally from inner–outer segment junctions towards the rod–bipolar synapses.

CONTROL OF THE DARK CURRENT BY LIGHT

If the ionic composition of rods is sufficient to account for their ability to produce electric currents, it is not surprising that cell differentiation should lead to an asymmetrical distribution of the current on the plasma membrane. Many epithelial layers, such as frog skin and toad bladder, show asymmetrical currents that arise in their constituent cells. Nevertheless, the dark current of retinal rods is remarkable in two ways. First, the current is large: it is sufficient to turn over all of the cations in a rat rod in about 1 min or in about 8 min in a larger frog rod (Hagins *et al.* 1970). Secondly, a single photon absorbed in an outer segment can suppress about 3% of the dark current (Penn & Hagins 1972) and produce a corresponding hyperpolarization of the cells (Baylor & Hodgkin 1973). If this effect were caused by the opening of a single ionic channel in the plasma membrane (perhaps a part of the rhodopsin molecule that was excited), the channel would have to be very large indeed. From the data of Table 1, the production of a light-activated outward K^+ current equal to 3% of the inward Na^+ dark current should require a channel whose unit conductance, g_c, would exceed $0.03I_D/(E_m-E_K)$. If E_m is equal to E_{Cl}, and I_D is of the order of 50 pA for a rat rod, g_c would have to be 150 $p\Omega^{-1}$ or more. Although unit conductances of this size or larger have been seen in some model systems (e.g. see Ehrenstein *et al.* 1970) they are unusual and imply an inefficient way for a cell to use its resources to convert light signals into changes in membrane

potential. A squid photoreceptor can produce single photon responses with light-induced Na^+ conductance increases of only 20 $p\Omega^{-1}$ (Hagins 1965).

Much evidence now indicates that vertebrate rods and cones do not operate by such an inefficient scheme. Instead, intracellular measurements of the membrane resistance of rods and cones indicate that light decreases their ionic conductance, probably to Na^+ (Baylor & Fuortes 1970; Tomita 1970). This conclusion is supported by the observation that light decreases the rate at which the dark-voltage gradient in the receptor layer collapses on poisoning with ouabain (Yoshikami & Hagins 1973), an effect not readily accounted for if the specific action of photons were to increase the K^+ conductance of any part of the cells.

INTRACELLULAR TRANSMITTERS AND CONTROL OF THE MEMBRANE CONDUCTANCE

The need for a mechanism by which light can suppress a large ionic conductance introduces the first of three problems concerning the excitatory process in vertebrate photoreceptors, namely, the numerical gain problem, the topology problem and the kinetic problem.

The numerical gain problem arises from the fact that a photochemical change in just one of more than 30 million rhodopsin chromophores produces a transient decrease of more than 3% in the resting 50 pA dark current of a rod (rat rods). The total effect of a single absorbed photon is the suppression of the flow of more than two million electronic charges (Hagins *et al.* 1970). Thus, a single photon produces an electrical effect with a charge amplification of more than 10^7. The 'molecular gain' of the light response $[(3/100)/(3 \times 10^7)^{-1}]$ (i.e. the ratio of the fractional change in dark current to that in rhodopsin content of a rod in a photon response) is also about 10^6.

The topology problem applies only to some kinds of vertebrate outer segments—those that have their photopigment concentrated in internal membranous disks. Electron microscopic evidence indicates that the disks of many rods are not continuous with the plasma membrane (Cohen 1970). Measurements of the electrical capacitance of the plasma membranes of rat rods (Penn & Hagins 1972; Rüppel & Hagins 1973) support this conclusion. Still a third way to show that disks of rods are detached from the plasma membrane but those of cones are not is by use of fluorescent staining with *NN'*-didansylcystine (DDC) (Yoshikami *et al.* 1974). As this fluorochrome binds to the lipoprotein surfaces of cells, phospholipid vesicles and micelles, its fluorescent efficiency is simultaneously much enhanced. However, it penetrates cell membranes only very slowly. When a live frog retina is stained

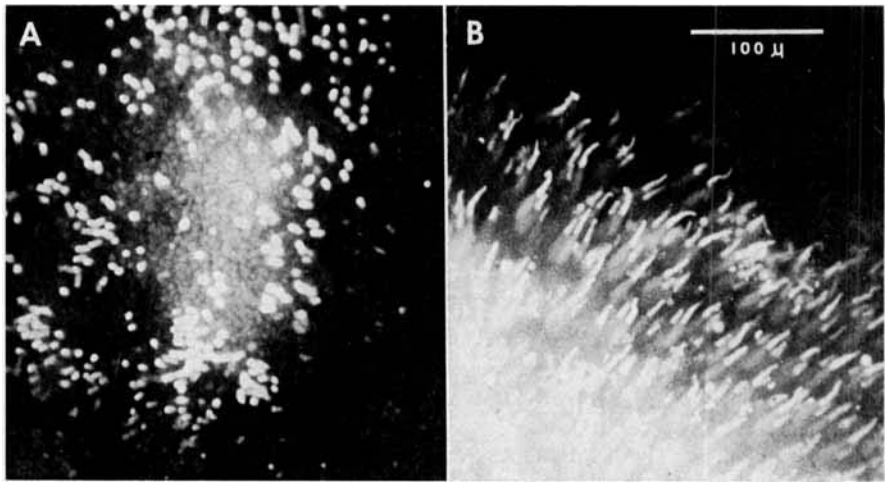

FIG. 5. Differences in the topologies of retinal rods and cones shown by differential staining of their outer segments with *NN'*-didansylcystine. (A) Flat preparation of fresh frog retina (*R. pipiens*) briefly exposed to 50% hypotonic Ringer's to rupture some of the plasma membranes of rod outer segments then stained with 10μM-didansylcystine for 2 min (observed by fluorescence microscopy): excitation, 365 nm, 2×10^{16} photons cm^{-2} s^{-1}; fluorescence emission: wavelengths $>$ 500 nm. The lack of staining of most outer segments indicates that the rhodopsin-bearing disks are shielded from the stain by an intact plasma membrane. (B) Fresh goldfish retina stained as above but without hypotonic shock. All cone outer segments are stained; thus, they are accessible to the stain. Nevertheless, the mitochondria in the inner segments are not stained, thereby showing that the plasma membranes of cones are impermeable to the stain.

with 10μM-didansylcystine, some of its damaged rod outer segments with permeable plasma membranes become brightly fluorescent owing to the dansyl derivative bound to the disks, but most do not (Fig. 5A). However, all the cone outer segments of a live goldfish retina immediately fluoresce brightly in 10μM-didansylcystine in Ringer's (Fig. 5B). Yet, if the rhodopsin-bearing disks of rods are detached from the plasma membrane, how do photons absorbed in them affect the dark current?

The kinetic problem arises from the observation that the waveform of the reduction in the dark current in response to a short stimulus flash is slow with a sigmoidal rise and slow exponential decay. Though it can be represented analytically by the formalism of chemical kinetics, none of the kinetic parameters bears any simple relation to the rates of the thermal reactions in the decay scheme of illuminated rhodopsin *in vivo* or *in vitro*. Thus, some additional time-consuming processes must link the primary photochemical event with the electrical one at the plasma membrane (Fuortes & Hodgkin 1964; Penn & Hagins 1972; Hagins 1972).

These three problems can all be resolved by supposing that the result of photochemical processes in the rod disks is the release of a diffusible intracellular transmitter that carries signals from a bleached rhodopsin molecule to the plasma membrane. If so, signal-to-noise considerations suggest that many transmitter molecules—perhaps 100 or more—are released by a single absorbed photon (Yoshikami & Hagins 1973; Cone 1973). The release, diffusion and reaction of the transmitter with the plasma membrane might then provide the necessary complexity to account for the kinetic form of the electrical responses of rods and cones.

THE IDENTITY OF THE INTERNAL TRANSMITTER

Although there are many possible intracellular transmitters, the topological problem is most economically solved by supposing that the transmitter is a normal constituent of the extracellular fluid but is normally excluded from the cytoplasm, except during the light response. Because Ca^{2+} ions are known to serve such transmitter functions in muscle and elsewhere, Ca^{2+} seems a likely candidate. Arguments and evidence in its favour are given elsewhere (Hagins 1972; Yoshikami & Hagins 1973; Hagins & Yoshikami 1974), but its role has neither been experimentally confirmed or disproved yet.

Evidence in favour of Ca^{2+} consists of four findings: (1) increasing the external $[Ca^{2+}]$ quickly and reversibly reduces the dark current. (2) Elevated external $[Ca^{2+}]$ reduces the rate of decline in the dark voltage gradient induced by ouabain in experiments like that shown in Fig. 4. Evidently the action of Ca^{2+}, like that of light, is to reduce the Na^+ conductance of the plasma membrane of rods. (3) The antibiotic X 537A, a specific ionophore for bivalent cations, greatly sensitizes the dark current to external Ca^{2+}. Although 20mM-Ca^{2+} is needed to suppress the dark current in normal rods, 10μM-Ca^{2+} suffices in rat rods exposed to 10μM-X 537A. Thus, applied Ca^{2+} seems to work *inside* the plasma membrane. (4) When rods are exposed to a solution with a low Ca^{2+} activity ($< 10^{-7}$ mol/l), they still maintain a substantial dark current but they are greatly desensitized to light, as if their internal store of transmitter is reduced or is less effective at the plasma membrane.

The desensitizing effect of solutions with low Ca^{2+} activity is complex but one important contribution is an apparent increase in the buffering capacity of the intracellular space for the transmitter. This can be shown simply by comparing the dark-current-reducing effect of a small test flash at different intensities of a superimposed steady background light. At Ca^{2+} ion activities, A_{Ca}, greater than 1 μmol/l in the bathing Ringer's, the effect of an applied background is to reduce the peak response to a test flash.

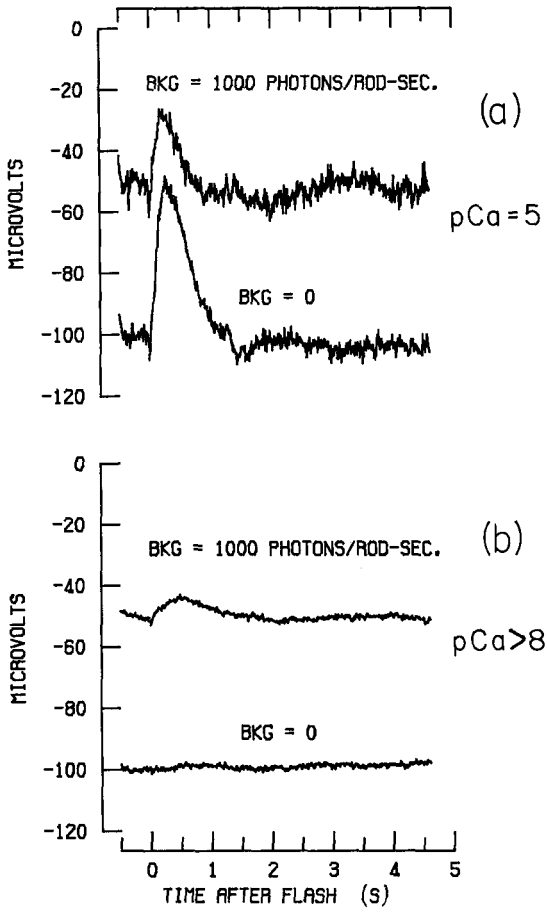

FIG. 6. The 'priming' effect of light on the photocurrent responses of rat rods at low Ca^{2+} activities. Extracellular recordings of the voltage differences between two Ringer filled micropipettes, one at the rod tips and one 80 μm deep in the receptor layer; flow chamber at 37 °C. The electrodes were placed under infrared visual control; stimulus wavelength 560 ± 10 nm. Each curve is the average of 8–32 responses.

(a) Reduction in the dark voltage gradient by 2 μs stimulus flashes causing about 15 photon absorptions per rod per flash on zero background and on a background equivalent to about 1000 photon absorptions $rod^{-1}\ s^{-1}$; Ca^{2+} activity 10 μmol/l (Ringer II of Hagins *et al.* 1970 with added EGTA). The background light reduced both the steady voltage gradient in the receptor layer and the size of flash responses in agreement with the hyperbolic saturation relation of Penn & Hagins (1972). (b) Same retina as in (a) but Ca^{2+} activity reduced to < 10nmol/l by substitution of a Ringer's containing about 2μM-Ca^{2+} (as impurities in other salts) and 1mM-EGTA. Flash responses on background of 1000 photons absorbed $rod^{-1}\ s^{-1}$ are now larger than if the background is absent.

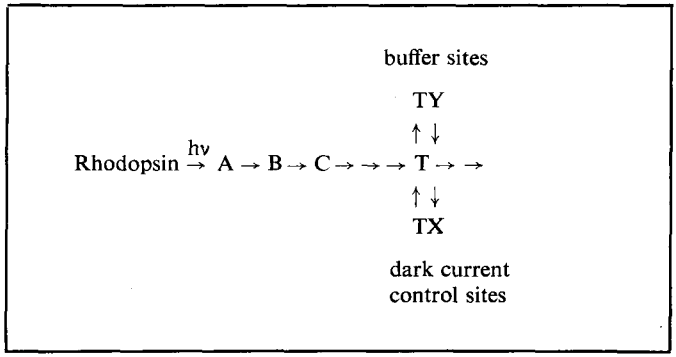

FIG. 7. Scheme for excitation (modified from Penn & Hagins 1972).

As described before (Penn & Hagins 1972), this reduction is monotonic with increasing background intensity and follows a hyperbolic saturation characteristic. (Professor Hodgkin has pointed out to us that the kinetics of the flash response are somewhat faster when the background is present.) If the Ca^{2+} activity in the Ringer's is reduced to less than 10 nmol/l, however, the flash response is increased in size by the background. This 'priming' effect of light is large and persists for tens of seconds after the background is removed.

A possible interpretation of the priming effect (Fig. 6) at low Ca^{2+} activities is that Ca^{2+}-binding sites Y lie within the outer segments that are fully saturated at external Ca^{2+} activities, A_{Ca}, greater than 1 μmol/l. When A_{Ca} is lowered to 10 nmol/l, however, sufficient Ca^{2+} leaves the outer segments to bare these sites and allow them to buffer the Ca^{2+} released from the disks by light. In the suggested formalism for excitation (Penn & Hagins 1972), this effect could be represented by the scheme in Fig. 7. In terms of such a model, the buffering capacity of the hypothetical binding sites are equivalent to the transmitter released by about 200 photons absorbed rod^{-1} s^{-1}.

Arguments such as these point to a transmitter function for Ca^{2+} but they are indirect and need further tests. The most conclusive test would be the demonstration of light-induced release of Ca^{2+} from isolated rod disks. At present, two such positive reports have been published; Mason *et al.* (1974) have measured ^{45}Ca fluxes from disk membrane vesicles and Szuts & Cone (1974) have studied net Ca^{2+} contents of hypoosmotically shocked rods in light and dark. Our own experiments with ^{45}Ca have not disclosed any such effects and we remain cautious about their interpretation at present.

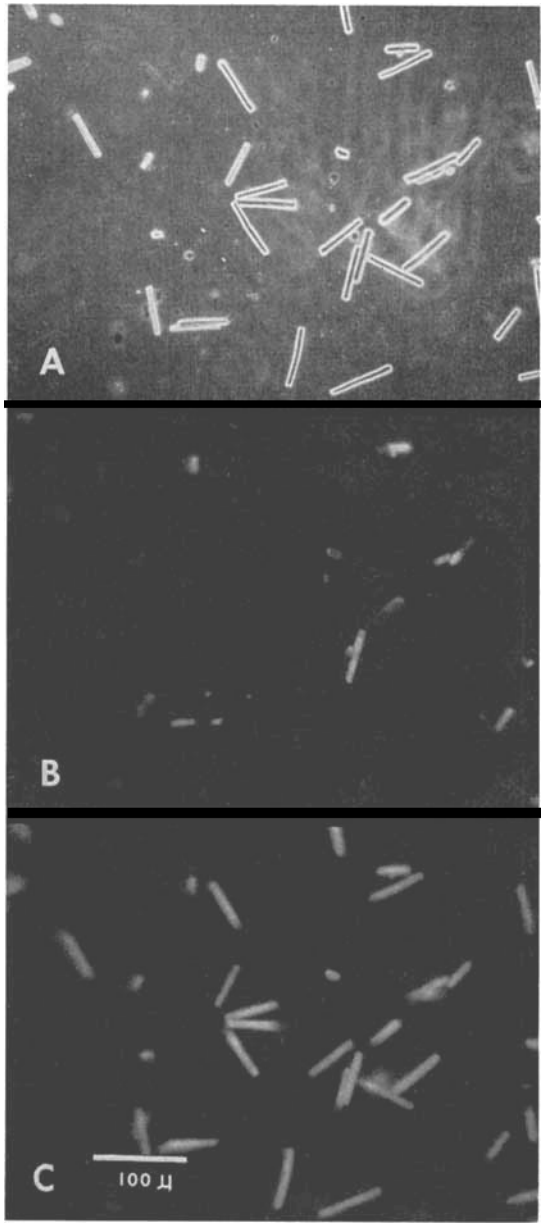

FIG. 8. Intactness of the plasma membranes of isolated frog (*R. pipiens*) outer segments embedded in 1% agarose Ringer's and washed with 10μM-*NN'*-didansylcystine in frog Ringer's. (A) Dark field micrograph; many rods of various lengths and shapes are visible; (B) same field by fluorescence microscopy (same optical conditions as Fig. 5)—many didansylcystine-permeable outer segments are visible; (C) same field as in (B) but after 2 min exposure to hypotonic (50% isosmotic) didansylcystine Ringer's—now all outer segments bind the stain.

FUTURE APPROACHES

Whatever excitatory transmitter process operates in rods and cones, its study requires new methods of studying biophysical processes in isolated outer segments, particularly the ionic permeabilities of disk and plasma membranes and their energy metabolism. Unresolved difficulties exist here, for isolated rod outer segments are not stable structures like erythrocytes. This can be shown by the *NN'*-didansylcystine staining test. The isolated frog outer segments seen by dark field microscopy (Fig. 8A) all look like uniform refractile bodies, yet when stained and examined by fluorescence (Fig. 8B) many are seen to have permeable plasma membranes. Studies of the staining process in isolated single outer segments (Yoshikami *et al.* 1974) show that the plasma membrane becomes permeable suddenly, like the bursting of a soap film. In some rods, such as that seen at the lower centre of Fig. 8B, the plasma membrane seems to have contracted so as to include a central group of protected disks, leaving bare ones at either end of the outer segment. Exposure of the outer segments to hypotonic Ringer's causes most of them quickly to become permeable to didansylcystine but only if they are embedded in agarose jelly. Freely floating outer segments often simply round up inside an intact plasma membrane to form refractile spheres. In view of this unstable behaviour, it is not surprising that work on the ionic permeability of isolated outer segments is progressing slowly (for example see Cobbs & Hagins 1974; Korenbrot & Cone 1972). Perhaps it is fair to say that the study of visual excitation *in vitro* is at about the same stage that oxidative phosphorylation reached with the rediscovery of the cytochromes by Keilin in the 1920s. We eagerly look forward to the discoveries of the next few years.

ACKNOWLEDGEMENT

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Discussion

Wilkie: The outer segments of the rods or cones are connected by a small canal with the rest of the rod or cone. When they are broken off, is a small hole left?

Hagins: Surprisingly not. If we wash a suspension of outer segments of the rods once, we can obtain rods about 30% of which have intact plasma membranes, to judge by their fluorescence in the *NN'*-didansylcystine technique (Yoshikami *et al.* 1974). When we analyse these for ATP by passing them through an ultrasonic mill and thence into an extract of firefly tails, we see a continuous glow of light, the intensity of which is proportional to the amount of ATP. Thus, at least some outer segments seem to be able to retain ATP unless subjected to sonication. This is a second piece of evidence that their plasma membranes are largely intact. Incidentally, the initial ATP concentration in freshly isolated outer segments usually exceeds 3 mmol/l of cytoplasm of didansylcystine-impermeable fragments.

Wilkie: Do you envisage a high current density in this narrow neck that joins the two segments?

Hagins: Yes, and the voltage gradient there should be of the same order as that in an electrophoresis cell, that is, a few V/cm.

Keynes: What are the energetics of the flow of this large dark current? How does the oxygen consumption compare with the amount of electrical work?

Hagins: Our measurements of electrical work done in sustaining the dark current carry an error of a factor of two because, in calculating the current, we must estimate the conductance of the plasma membrane. If that is allowed for, the Q_{O2} for the oxygen consumption (mm^3 O_2 gas at STP consumed per h per mg of tissue dry wt.) is between 25 and 50, a range which is much higher than anybody has previously recorded by direct respirometry.

Keynes: Couldn't you do direct calorimetry, because the production of heat must be high?

Hagins: That would be a good test.

Hastings: Judging from the current density, would you expect much ATP to be used in these cells?

Hagins: Yes, but the problem is, where is the ATPase that drives the dark current? It could be in the inner segment which does not break off. If so, how does the cell keep its ATPase segregated on the inner segment of the membrane? Clearly, rods do know how to do this since rhodopsin is found in the plasma membrane of the outer segment but not in that of the inner segment (Rüppel & Hagins 1973).

Hastings: Do you measure the amount of ATP in the whole cell?

Hagins: We just measured the outer segments and found the concentration exceeded 3 mmol/l there. We were surprised that they used the ATP as slowly as they did. The half-time for ATP consumption is about 30 min for frog rods at 25 °C. Possibly, something inhibits the ATPase(s) in the outer segments; the enzyme(s) isn't necessarily absent there.

Keynes: No objection can be raised to any asymmetric distribution of the Na^+,K^+-ATPase; after all, every cell in a secretory epithelium has the enzyme located differently on the two sides of the cell.

Hagins: In a sense, the rod layer *is* a piece of secretory epithelium.

Glynn: Wouldn't you want the ATPase to be on the outer segment, if it is that segment which pumps sodium out? Which way do you want the current to flow?

Hagins: If it is an electroneutral pump, it would be effective on any part of the plasma membrane, but most effective on the cell body or inner segment.

Huxley: What is known about the energy transformation? What happens in the rhodopsin molecule?

Hagins: The electronic transitions of the rhodopsin molecule are unusually well coupled to photochemistry. Rhodopsin does not fluoresce; the one report of its fluorescence (Guzzo & Pool 1967) has not been confirmed (Busch *et al.* 1972). No intermolecular energy transfer between rhodopsin molecules has been detected, probably because of the lack of fluorescence (Hagins & Jennings 1959). Consequently, each rhodopsin molecule acts as a single isolated individual.

Hess: With each photon, about 210 kJ are absorbed by the molecule. How is that energy consumed?

Hagins: Thermodynamically, the input of 1 kJ of light into rhodopsin is converted into 1 kJ of heat (apparently,—we have a 10% experimental error) (Hagins & McGaughy 1967 and unpublished results; see also Falk & Fatt 1972).

Huxley: Does the individual rhodopsin molecule act directly as an ion gate as well as a light catcher?

Hagins: The only evidence for that is negative. Electrophoresis of as pure rod outer segments as possible on a sodium dodecyl sulphate gel gives no other band besides the 'heavy' rhodopsin band and a light band (molecular weight 220 000), which may be an impurity or another constituent of the rod. In other words, these rods contain minimal macromolecular machinery; outer segments seem to consist of phospholipids, rhodopsin and possibly one other component. They do not burn ATP fast, nor do they seem to do anything else, but somehow they act as the transducers that allow vision. Thus, it seems possible that the rhodopsin molecules might act directly as ion gates, since there seems to be no other protein around to serve this function.

Glynn: Are the rhodopsin molecules attached to the membrane?

Hagins: Yes. They always centrifuge down in the membrane fraction of cell brei; they are closely associated with phospholipid.

Oesterhelt: Does the light energy absorbed merely isomerize 11-*cis*-rhodopsin to the all *trans*-form or does it do more to the protein?

Hagins: No evidence supports the idea that any other photochemical reaction of appreciable quantum efficiency ensues when rhodopsin absorbs photons, so there is no reason at present to suppose that light has any other excitatory action apart from isomerization. Isomerization does convert rhodopsin into a completely different kind of protein (as judged by a variety of biochemical tests). However, the changes consequent on the absorption of light depend on the state of the rhodopsin. In rhodopsin solutions in mild detergent, for example, the absorption of the photon considerably alters the circular dichroism spectrum in the 200–300 nm region. The number of accessible thiol groups and the binding of protons is also affected by bleaching in detergent solutions.

Huxley: Are these changes the consequences of isomerization, or are both the isomerization and these changes in the protein induced by the absorption of a photon?

Oesterhelt: That is exactly my point. Opsin must have a conformational state of higher energy than rhodopsin has and this energy has to come from absorbed light energy and not from the isomerization of retinal. Otherwise, one could not understand how regeneration of rhodopsin from opsin and 11-*cis*-retinal occurs as an irreversible reaction.

Hagins: The 'trigger' reaction might produce no net change in the heat content of the system, except for the energy in the photons, since all that is necessary for a reliable excitatory system is for an absorbed photon to pump a molecular system from one energetic valley, representing rhodopsin with an 11-*cis*-chromophore, over a hill much higher than kT to another valley representing rhodopsin with an all *trans*-chromophore but with the same energy. From this valley, however, further thermal reactions could occur. The energy of the absorbed photon might thus be 'conserved' in pushing a rhodopsin molecule 'up the hill' but be released and degraded in going down the other side to the bleached state. Since the techniques are available, it would certainly be worthwhile to determine the heats of reaction in these processes.

Roseman: But how does one photon trigger this huge effect? One molecule of certain colicines causes the same effect on the transport of certain sugars in the whole cell. Possibly, the effect on one protein molecule in a membrane spreads wave-like to influence the whole array of lipids in the membrane. Just by rearrangement of the lipid molecules with respect to each other, a

single event could be magnified countless times, thereby altering, for example, the permeability of the membrane.

Huxley: But what does happen in photochemical systems?

Oesterhelt: Opsin has to conserve some of the energy derived from light. Light converts rhodopsin into prelumirhodopsin (in the all *trans*-configuration) which gives by an irreversible reaction, all *trans*-retinal and opsin (see Fig. 1). The free-energy change for the conversion of all *trans*- into 11-*cis*-retinal is 5.0 kJ (Hubbard *et al.* 1965). Opsin can react irreversibly with 11-*cis*-retinal to form rhodopsin. Thus, light must provide at least −20.2 kJ for the cycle (see Fig. 1).

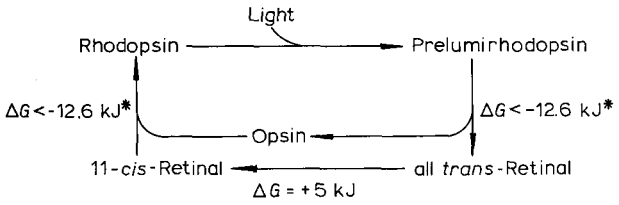

FIG. 1 (Oesterhelt). Free-energy changes for the photoreactions of rhodopsin.

* Calculated for equilibrium constants of at least 10^3 to account for the irreversibility of these two processes.

Huxley: Is that reversal spontaneous or does it require metabolic energy to drive it?

Oesterhelt: Rhodopsin can be reconstituted in a test tube.

Hodgkin: Is there any reason to believe that the opsin in prelumirhodopsin has the same free energy as the opsin that combines with 11-*cis*-retinal to form rhodopsin?

Oesterhelt: According to the literature, yes!

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The ionic channels in excitable membranes

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Abstract The ionic channels in excitable membranes are of two classes: those that open and close when the membrane potential alters and those that respond to the release of an appropriate chemical transmitter. The former are responsible for the conduction of impulses in nerve and muscle fibres and the latter for synaptic transmission. It is now clear that the sodium and potassium channels in electrically excitable membranes are functionally distinct, since each can be blocked without affecting the behaviour of the other. It has recently proved possible to study, in the voltage-clamped squid giant axon, the movements of the mobile charges or dipoles that form the voltage-sensitive portion of the sodium channels, which give rise to the so-called 'gating' current. Detailed comparisons can now be made between the kinetics of the ionic conductances as described by Hodgkin & Huxley, and the steady-state distribution and kinetics of the charged controlling particles, which should lead to useful conclusions about the intramolecular organization of the sodium channels and the conformational changes that take place under the influence of the electric field. There is as yet little information about the chemical nature of the electrically excitable channels, but significant progress has been made towards the isolation and characterization of the acetylcholine receptors in muscle and electric organ.

The major landmark for electrophysiologists in recent years has been the analysis by Hodgkin & Huxley (1952) of the mechanism of nervous conduction in the squid giant axon in terms of a sequence of time and voltage-dependent changes of the permeability of the nerve membrane to sodium and potassium ions. They postulated that the sodium conductance, g_{Na}, was governed by the equations (1)–(3) where $\bar{g}_{Na}$ is a constant representing the peak sodium con-

$$g_{Na} = \bar{g}_{Na} m^3 h \tag{1}$$

$$\frac{dm}{dt} = \alpha_m(1 - m) - \beta_m m \tag{2}$$

$$\frac{dh}{dt} = \alpha_h(1 - h) - \beta_h h \tag{3}$$

ductance per unit area, m is a dimensionless variable ($0 < m < 1$) representing the proportion of controlling particles in an activating position, h is a similar variable for the inactivating particles, and the α's and β's are transfer rate constants for forward and backward movements, which vary with voltage but not with time. Similarly, equations (4) and (5) may be written for the potassium conductance, g_K, where n is another dimensionless variable. Equations of this form have since been shown to describe the changes in ionic conductance

$$g_K = \bar{g}_K n^4 \tag{4}$$

$$\frac{dn}{dt} = \alpha_n(1 - n) - \beta_n n \tag{5}$$

with excellent fidelity for a wide range of electrically-excitable tissues.

It has sometimes been proposed that the conductance changes all take place in a single set of ionic channels, which are selective for sodium on first opening but later revert to a potassium-selective state. This idea has now been dropped in favour of a concept of two entirely-distinct classes of channel. The most telling piece of evidence is that the potassium channels can be completely blocked by quaternary ammonium or caesium ions without in any way affecting the kinetics of the sodium conductance change, whereas precisely the converse is true for the blocking of the sodium channels by tetrodotoxin. It is hard to see how a single channel could behave in this fashion.

An essential feature of any molecular model devised to account for permeability changes of this kind is that it must allow for voltage-sensitivity. The conductance changes must in some way be linked with a movement of mobile charges or dipoles within the membrane under the influence of the electric field. A highly diagrammatical representation of a model of a sodium channel that is compatible with equations (1)–(3) and some, but not all, of the new experimental findings that I shall discuss is shown in Fig. 1. In this, the aqueous channel is surrounded by globular gating particles which behave as dipoles free to rotate in the electric field. In the resting state, the three particles labelled m are oriented so that their positive changes lie close to the inner end of the channel and block the passage of cations through it. When the field is reversed, the m particles rotate through 180°, and as soon as all three are in their new position, cations can traverse the channel. The single triply-charged h particle that is responsible for inactivation rotates more slowly, but blocks the channel once more when its positive charges reach a position close to the outer end. The selectivity of the channel towards Na^+ ions depends on the presence of a funnel-shaped filter located on the outside, in series with the gating mechanism.

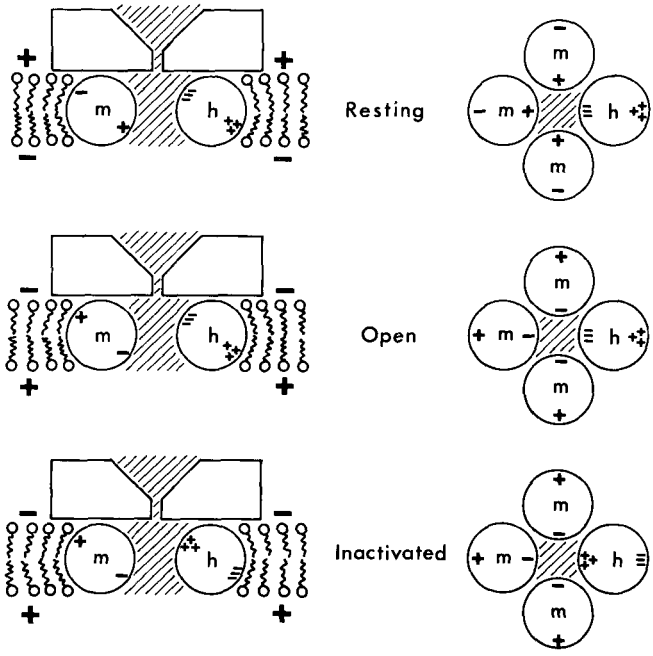

FIG. 1. Portrait of a sodium channel based on a model proposed by Rojas (1975).

This model should on no account be taken too literally, since it simply serves to indicate the general kind of system with which we are concerned.

Whether the mobile charges of the gating system are disposed as in Fig. 1 or are arranged in some other way, an opening of the gate will involve an outward transfer of charge. When, therefore, the membrane is depolarized, the resulting flow of ionic current is preceded not only by the surge of capacity current but also by what has come to be called the 'gating current'. Although the existence of this component of the membrane current was predicted by Hodgkin & Huxley (1952), its very small size relative to the ionic current prevented them and, later, Chandler & Meves (1965) from detecting it experimentally. Only in the last year or two, with tetrodotoxin being used to block the sodium current, has it become possible to unmask the gating current (Armstrong & Bezanilla 1973, 1974; Keynes & Rojas 1973, 1974; Meves 1974) and to begin to examine its characteristics.

The asymmetry of the displacement current on application of depolarizing and hyperpolarizing voltage-clamp pulses of equal size is shown in Fig. 2, where the top pair of traces are single-sweep records at high gain of the membrane current for $\pm$ 120 mV pulses applied to an axon in which the sodium and

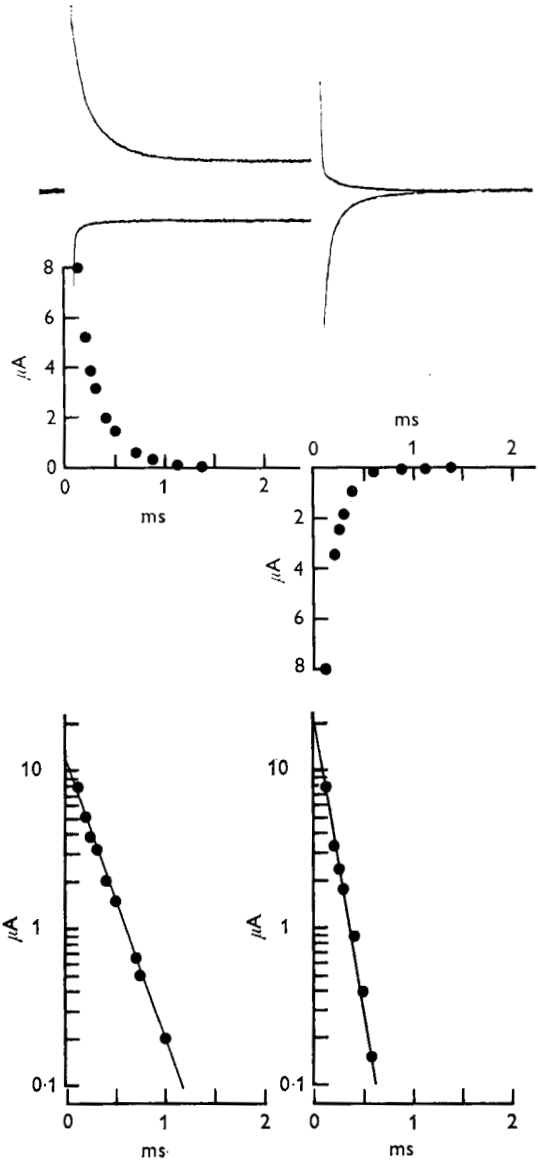

FIG. 2. Asymmetry of the displacement current on application of equal and opposite voltage-clamp pulses to a squid axon perfused with 300mM-CsF and bathed in Na- and K-free saline containing 1000nM-tetrodotoxin. The top traces are single-sweep records of the membrane current for ± 120 mV pulses. The difference between them is plotted beneath on linear and logarithmic scales. (Fig. 4*B* from Keynes & Rojas 1974).

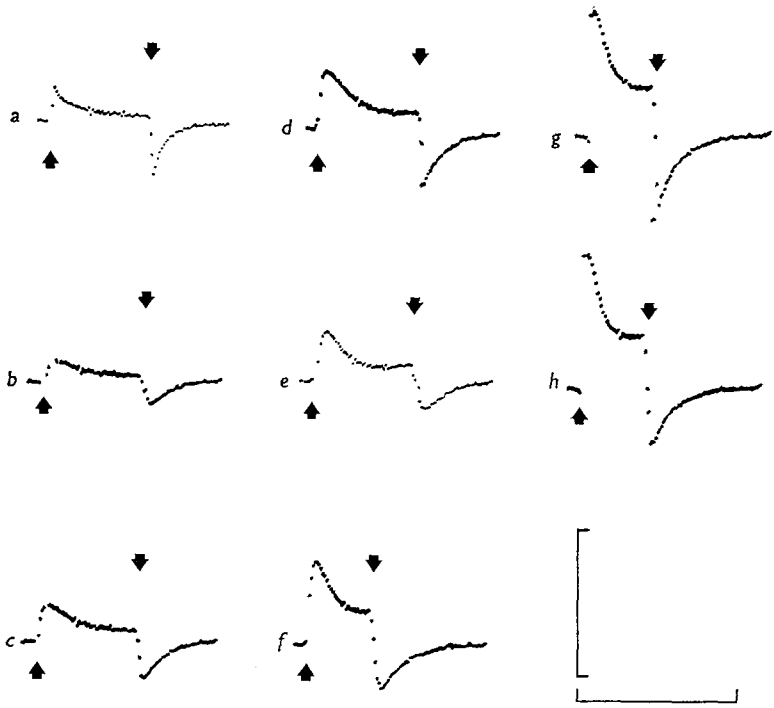

FIG. 3. A family of gating current records obtained by summation with a signal averager of the membrane currents for 60 positive and 60 negative pulses which started and finished at the arrows. The axon was perfused with 55mM-CsF, and bathed in Na- and K-free saline containing 300nM-saxitoxin: pulse amplitude, *a–h*, 40–110 mV; holding potential −70 mV; vertical bar 5.56 μA, horizontal bar 2500 μs; membrane area 0.06 cm²; temperature 7 °C (Fig. 6 from Keynes & Rojas 1974).

potassium currents have been effectively blocked. After the capacity transient is complete there is a slow tail of outward current at the beginning, and of inward current at the end, of the positive pulse but not of the negative one. Addition of the records reveals, as in the lower plots, that the asymmetrical component has an exponential time course.

Our normal procedure for recording the asymmetrical displacement current was to use an analogue signal averager to do the addition and at the same time to improve the signal/noise ratio. A typical family of gating current records is shown in Fig. 3. In this axon, as in most of the others, there was some rectification of the anionic leakage current during the pulse, which resulted in the superimposition of a rectangular pedestal on the exponential tails.

The fundamental criterion for regarding the sharply rising and exponentially

declining components of the asymmetrical displacement current as due to the movement of mobile charged particles or dipoles that form an integral part of the membrane is that the total transfer of charge in one direction at the start of the pulse should be exactly equal to that in the opposite direction at the finish. This we have shown (Fig. 8 in Keynes & Rojas 1974) to be satisfied within the accuracy of our measurements. Another essential condition is that the charge displacement should reach a saturation level when large enough pulses are applied to the membrane; and this too is met (Keynes & Rojas 1974; Fig. 9). A further argument is that lowering the temperature at which the observations are made reveals no change in the total amount of charge transferred for a pulse of a given size, although the time constants of the exponentials have a large temperature coefficient. There thus seems no doubt that the observed asymmetry arises from the presence of mobile membrane charges of some kind, and the question we have to decide is whether they can be identified with the sodium gating particles.

One piece of evidence that supports such an identification is illustrated in Fig. 4, in which normalized values of the time constants of the asymmetrical displacement currents recorded in a number of experiments have been plotted against membrane potential. The solid line represents a possible theoretical relationship that may be based on too simple premises to have great significance, although the fit could be worse. The dashed line is the empirical curve used by Hodgkin & Huxley (1952) to describe their data for the dependence of τ_m (which equals $[\alpha_m + \beta_m]^{-1}$, where α_m and β_m are the rate constants in equation 2) on potential. Both in absolute magnitude and in voltage dependence, there is good agreement between τ_m and the relaxation time constants of the mobile charges. This agreement also extends to the effects of changing the external calcium concentration and of perfusing the axons with solutions of low ionic strength, both of which actions shift τ_m and the relaxation time constants to much the same extent along the voltage axis without altering their size.

From an appropriate plot of the steady-state distribution of the mobile charges against potential, one can arrive at an estimate of their effective valency, which turns out to be 1.3; an *e*-fold change is caused by 19 mV alteration in potential (Keynes & Rojas 1974). The corresponding figure for an *e*-fold change in the sodium conductance of the membrane is 6 mV. This suggests some kind of cooperative mechanism, the simplest interpretation being that there are three gating particles per channel. This is, of course, precisely the conclusion reached by Hodgkin & Huxley (1952) from their data for the kinetics of activation of the sodium conductance, which led them to write m^3 in equation (1) rather than m^2 or m^4.

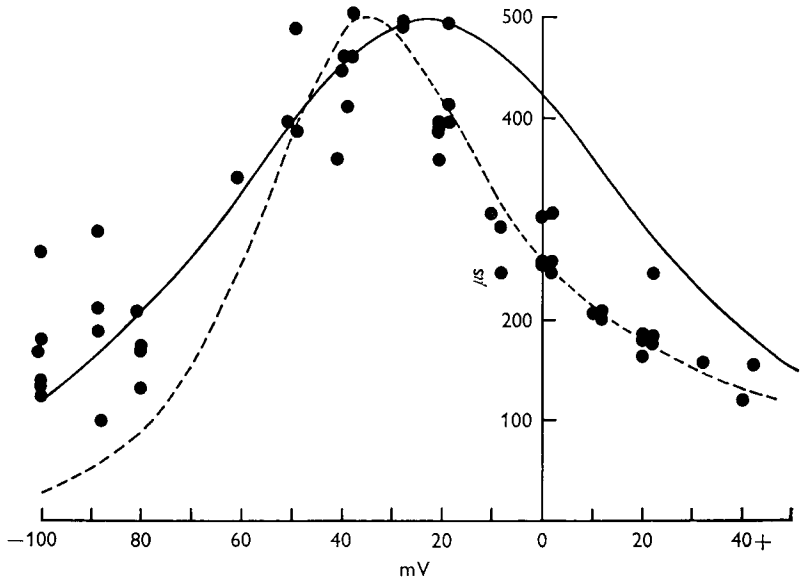

FIG. 4. Normalized values of the gating current time constant (in μs) at 6 °C plotted against membrane potential (in mV): the solid line represents equation (18) of Keynes & Rojas (1974) and the dashed line shows the value of τ_m from equations (20) and (21) of Hodgkin & Huxley (1952). (Fig. 20 from Keynes & Rojas 1974).

Although, therefore, we have good reasons for believing that the asymmetrical displacement currents that we observe arise from the movements of the hypothetical '*m*' particles of Hodgkin & Huxley, there are certain respects in which the behaviour of the gating current cannot be reconciled perfectly with equations (1)–(3). Although the gating current time constant at the beginning of a pulse, τ_{on}, fits well with τ_m calculated on an m^3 basis, τ_{off} at the end of the pulse fits less well, for the records of Armstrong & Bezanilla (1974) agree with some of our own (see Keynes & Rojas 1974) in indicating that the time constant for shutting off the sodium conductance is nearly equal to τ_{off}, instead of being only one third as great. However, incomplete compensation for the electrical series resistance when recording the sodium current can considerably shorten the conductance turning-on time constant and prolong that for turning-off. Subsequent records in which more attention was paid to this source of error (Rojas & Keynes 1975; Fig. 2) appear to show less of a discrepancy between $\frac{1}{3}\tau_{off}$ and the conductance turning-off time constant. A further difficulty is that, as we and Meves (1974) agree, τ_{off} is generally about twice as great for large voltage-clamp pulses as it is for small ones, but no such variation is seen in the turn-off of conductance. Nevertheless, it is not hard to think of ways in

which an interaction of the *m* particles after they have made their transition to the 'gate open' position might account for these differences. It is in their existence that the value of being able to record the gating current lies, and a careful comparison between the kinetics of the gating particles on the one hand and of the sodium conductance changes on the other, should in due course throw valuable light on the nature of the conformational changes.

Possibly the most important feature of observations on sodium gating currents is the prospect that they offer of unravelling the mechanism of inactivation. Here there is, unfortunately, a conflict between our results and those of Armstrong & Bezanilla (1974) that needs to be resolved. According to them, maintenance of the membrane potential at a positive value for a long period results in the disappearance of the gating current, which is thus inactivated in parallel with the sodium conductance; perfusion with 10mM-$ZnCl_2$ similarly cuts both down together. A weakness of their otherwise fairly convincing evidence is that so far they have published records only of the events at the beginning of voltage-clamp pulses, and none of the 'tails' at the end. The data in Fig. 5, which are fully confirmed by Meves (1974) despite a number of differences in technique, show that the squid in Plymouth seem to differ from those at Woods Hole by displaying not an absence of gating current on prolonged depolarization but a complete reversal of the signals. We believe, therefore, that the distribution of the gating particles on either side of the membrane is at all times determined by the prevailing electric field, and that they remain free to move even when the sodium conductance is completely inactivated. Inactivation should not, on this view, involve a freezing of the position of the gating particles as Armstrong & Bezanilla (1974) apparently propose.

What alternative is there? The most straightforward hypothesis is that the channels are blocked by the movement of a separate *h* particle in the manner indicated in Fig. 1. In this case, however, the fact that the h_∞ curve (Hodgkin & Huxley 1952) shows an *e*-fold change in 7 mV at its steepest point means that the effective valency of the *h* particles ought to be about 3.6. Taking Hodgkin & Huxley's values for the rate constants of the *h* process, we can then calculate that on pulsing the membrane to a potential of 0 mV, we should record a second component of the gating current that would initially be nearly quarter as great as the *m* current and would decline about quarter as fast. Although I certainly do not preclude the existence of small but slow extra components of the gating current that in the analyses made so far have been left buried in the pedestal of ionic leakage current, it seems unlikely that an *h* component of this size could have been missed. I am, therefore, inclined to dismiss the concept of an independent *h* particle in its simplest form. Instead,

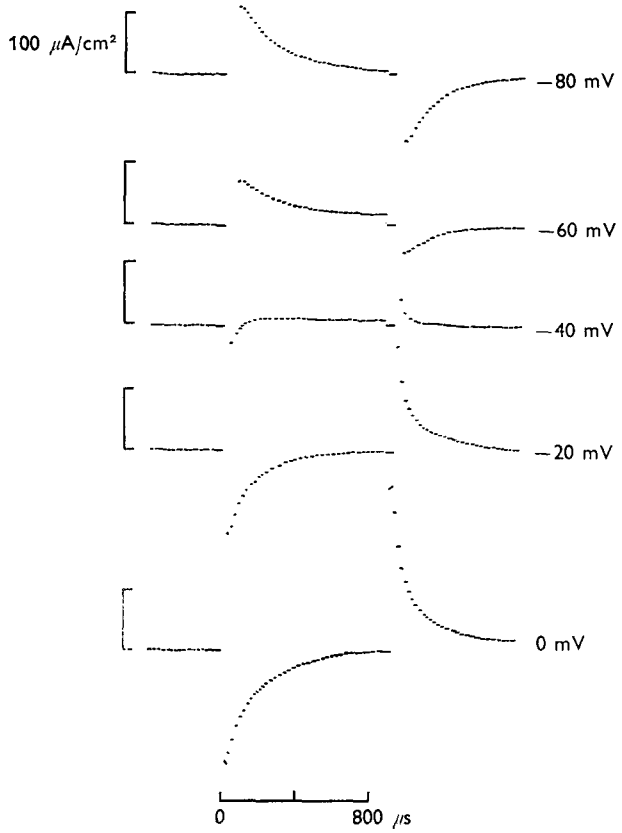

FIG. 5. The effect on the displacement current records of varying the holding potential. Axon perfused with a CsF solution of low ionic strength, and bathed in acetate–saline containing 300nM-tetrodotoxin: pulse size, ±150 mV. The membrane potential was held for several minutes at the level indicated to the right of each record (Fig. 1 from Keynes *et al.* 1974).

the possibility must be explored that the system consists purely of a set of activating particles, each of which may be in one of three states: resting, open, inactivated. Granted a free choice of the laws governing the transitions between these states, we can construct models that display the kinetics for sodium conductance described by equations (1)–(3). Here again, the principal value of the gating current measurements will be to provide a fresh set of constraints on the types of model that are compatible with the physical facts.

Another aspect of this evidence that needs to be mentioned concerns the value of the membrane potential at which the gating particles are equally distributed on the two sides of the membrane. From the analyses by Hodgkin

& Huxley (1952), the midpoint of the m_∞ curve lies at about –37 mV. In intact axons, the midpoint of our steady-state distribution curve for the gating current came out at –22 mV (Keynes & Rojas 1974). If we allow for some uncertainty in estimating the junction potentials in the system, these two figures are not too far apart; and the agreement between them has been confirmed in subsequent measurements. If our interpretations are correct, one might expect the potential at which the signals are inverted when the holding potentially is gradually lowered, as in Fig. 5, to be the same; but it often seems to be appreciably more negative, and in Meves's (1974) experiments was close to –60 mV. It is not at present clear what significance should be attached to this difference, but I suggest that it is related in some way to the mechanism of inactivation; it should not be forgotten that the midpoint of the h_∞ curve also falls at –60 mV. The relatively slow changes in the size of the gating current noted by Meves (1974) must also be taken into account. The kinetics of the variation in the transition potential are high on the priority list for more detailed investigation.

Little definite can be said about the precise chemical structure of the sodium channel. Presumably it is a protein, and the molecular weight of the tetrodotoxin-binding site has been determined by irradiation inactivation as 229 000 (Levinson & Ellory 1973). It is, however, uncertain whether the portion of the molecule involved in gating should be regarded as included within this estimate, since the selectivity and gating mechanisms are to some degree independent of one another. The location of the sodium filter in Fig. 1 on the outer side of the membrane, beyond the influence of the electric field, accords with the facts that the size and time course of the gating current are the same whether or not the sodium channels are blocked with tetrodotoxin (Armstrong & Bezanilla 1974; Keynes & Rojas 1974), and that tetrodotoxin and saxitoxin act only at the outside of the membrane. Hopefully, we shall be able to apply to the sodium channels some of the techniques for extraction and purification that have given such promising results with Na,K-ATPase (Jørgensen 1974) and acetylcholine receptors (Green *et al.* 1975), but the preparation of derivatives of tetrodotoxin and saxitoxin that could be used, for example, in affinity chromatography of the sodium channels is fraught with difficulty, and their proper chemical characterization is another important question for the future.

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Discussion

Huxley: It was a great relief to me and no doubt also to Professor Hodgkin when these currents turned up and more or less satisfied our predictions. I am certainly not surprised at the differences (see Fig. 4), for there was an element of speculation about the formulae to be fitted—others can be made to fit the data.

Weis-Fogh: How many gates are there in the squid giant axon compared with the rabbit vagus and other nerves? This links up with the situation which Hagins described for rods.

Keynes: The average value for the total displacement of charge we calculate at about 1800 charges μm^{-2}. We believe that there are three gating particles per channel and that the 19 mV (p. 196) corresponds to an effective valency of 1.3 (that is, the magnitude of the charge multiplied by the fraction of the field it moves through; we cannot be more precise than that). So, the total effective gating charge per channel will be 3.9 (3×1.3), in other words there are about 450 channels μm^{-2}. Armstrong & Bezanilla (1973) originally quoted a figure 10 times smaller, but they have corrected that, so that now our values do not disagree grossly. Two pieces of evidence support such a figure for the squid giant axon. First, Levinson & Meves (1975) have determined the amount of labelled tetrodotoxin that is bound, finding some hundreds of channels

μm^{-2}. Secondly, Rojas, Taylor & I have some unpublished results on the kinetics of tetrodotoxin which imply 300–400 channels μm^{-2}. The figure considerably exceeds that similarly obtained for other axons, for example 2.5 μm^{-2} for garfish olfactory nerve and about 25 μm^{-2} in rabbit vagus (Colquhoun *et al.* 1972). Professor Hodgkin has calculated the optimum number for maximum velocity of conduction as about 1000 μm^{-2}.

Huxley: What order for conductance per channel does this correspond to?

Keynes: About 2.5 pS ($p\Omega^{-1}$).

Hagins: For squid photoreceptors, the conductance of a light-induced Na^+ channel, from the shot noise in their photocurrent (Hagins 1965) is about 20 pS ($p\Omega^{-1}$), but since no other data exist for comparison, the accuracy of that value is unknown. We don't know how many channels vertebrate rods contain, so the figure could be higher.

Keynes: Hille (1970) quoted a substantially bigger figure, but he based his calculation on the assumption now seen to be false that the number of channels in unit area of the squid giant axon was the same as in a much smaller axon.

Hodgkin: Dr Hagins, do you share the view that there is substantial amplification in the transduction mechanism in rods?

Hagins: Yes, because I don't see how else one single calcium ion could act alone. If the calcium ion concentration in the cytoplasm of a rod were 1 μmol/l there would still be at least 10 000 free calcium ions, but at 10 nmol/l there would still be 100. Release of a single Ca^{2+} ion by a proton could not change $[Ca^{2+}]$ by 3%, yet a single proton can reduce the rod dark current by that amount.

Huxley: Had you considered an alternative to calcium?

Hagins: If it were something less common than Ca^{2+} ions in cytoplasm, amplification need not be necessary, but we do not have any particular substance in mind.

Wilkie: Professor Keynes, does tetrodotoxin block sodium channels if it is applied internally?

Keynes: Even large quantities of tetrodotoxin have no effect internally. The part of the sodium channel that is blocked by tetrodotoxin seems to operate independently of the part that is voltage-sensitive; tetrodotoxin leaves the gating currents unaltered. Tetrodotoxin seems to act in the aqueous phase outside the voltage-dependent part of the system.

White: If the *h* gates are just 10 times slower than the *m* gates, would you not expect to see some effects from them in your experiments?

Keynes: We would, but we don't. From the data of Hodgkin & Huxley (1952), we can calculate the expected inactivation or *h* gating current to be

about a quarter the size of and four times slower than the *m* gating current for pulses depolarizing down to about 0 mV.

All our records appear to be single exponentials, however, with no sign of a component with *h* kinetics. Since no *h* gating current can be detected, we shall have to fall back on a different type of explanation for inactivation. I have designed a model in terms of three possible energy states for the gating particles, but it is still at an early state of development.

Lipmann: Are there any electron micrographs of such channels and such particles?

Keynes: Unfortunately not. However, calculation from the above figures indicates that the channels would be few and far between in an electron micrograph of the membrane. Absence of the channels might then be merely the accident of a poor section.

Glynn: Is it possible that you do not detect the gating currents corresponding to the inactivating particle simply because, at the starting potential, that particle is sitting in a mid-position and can be displaced equally easily in either direction?

Keynes: This is the reason for the disappearance of the net displacement current in Fig. 5 at a starting potential of about –48 mV. However, the midpoint of the h_∞ curve is supposed to be at –60 mV, so that one could not account in the manner you suggest for the absence of *h* gating current when the starting potential is –100 mV.

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Different approaches to the mechanism of the sodium pump

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Abstract The way in which the sodium pump uses energy from the hydrolysis of ATP to perform osmotic and electrical work is not yet understood. We attempt to bring together the results of a number of different approaches to this problem.

One approach has been to correlate biochemical changes and ionic fluxes, both when the pump operates normally and when it operates in various abnormal 'modes' in particular unphysiological conditions. A second approach has been to expose fragments of cell membrane to [γ-^{32}P]ATP and to study the properties of components of the membrane that become labelled. It is now clear that ^{32}P can be transferred to the β-carboxy group of an aspartyl residue in a pump polypeptide, but there is controversy about the interrelations of different forms of this polypeptide and its role, if any, in the normal functioning of the pump. A third approach has been to attempt to purify the pump and to determine the properties of the pure enzyme. It seems that the pump contains a polypeptide (molecular weight about 100 000), which bears the phosphorylation site, and a smaller glycopeptide, but there is disagreement about the molecular ratios.

The results of these and other approaches cannot yet be fitted into a satisfactory model for the sodium pump, but we shall consider some of the problems involved in this task.

During the last few years information about the Na pump has come from a variety of different approaches; in this paper, we shall try to piece together some of this information.

The approach with which we have been mainly concerned is to see what fluxes the pump can cause in abnormal conditions, what the biochemical requirements for these fluxes are, and what biochemical changes are associated with them. By manipulating the conditions, we can now make the pump operate in five different modes (Fig. 1): the normal mode; a reversed mode in which the pump runs backwards, synthesizing ATP; a mode in which internal

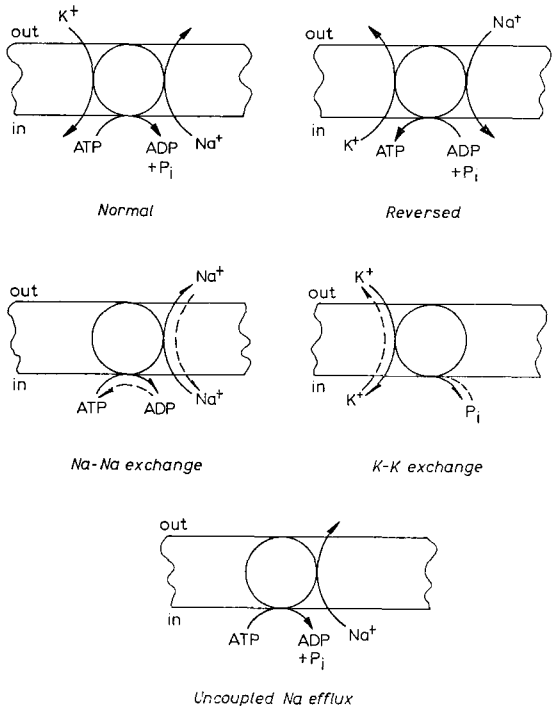

FIG. 1. The five modes of behaviour of the Na pump in intact red cells or resealed ghosts.

and external Na are exchanged; a mode in which internal and external K are exchanged; and a mode in which Na is expelled but not in exchange for either Na or K, so that we can speak of an uncoupled Na efflux.

The reversed mode and K–K exchange have been seen convincingly only in red cells, but they would not be easy to detect in other cells, and we have no reason to think that they cannot occur generally. Without a doubt, all five modes are caused by the same system: there are detailed resemblances in their responses to physiological ligands; all are inhibited by cardiac glycosides; and, with the exception of the reversed mode, which has not been tested, all are known to be inhibited by an antiserum to a partially purified preparation of the sodium pump from pig kidney (Glynn *et al.* 1974).

Under physiological conditions, in red cells (Sen & Post 1964; Whittam & Ager 1965; Garrahan & Glynn 1967*c*), in nerve (Bonting & Caravaggio 1963; Baker 1965) and in muscle (Dydyńska & Harris 1966), about three Na^+ ions are expelled for each molecule of ATP hydrolysed; and, in red cells at any rate, the stoichiometry does not depend on the work done in moving the ions. In this

respect, the pump seems to have a fixed gear. The number of potassium ions taken up is less than the number of sodium ions expelled and, for several excitable tissues, there is good evidence that the pump is electrogenic, that is to say that its activity leads directly to a net movement of charge across the membrane (Thomas 1972). In squid axons (Mullins 1972; Mullins & Brinley 1969), snail neurons (Kostyuk *et al.* 1972), red cells (Karlish & Glynn 1974) and perhaps also frog muscle (Adrian & Slayman 1966; Cross *et al.* 1965) there is evidence that the ratio between the movements of Na^+ and of K^+ can change with the conditions. In this respect, the gearing seems to be variable.

In red cells under physiological conditions the pump is about 70% efficient and, by arranging that the concentration gradients for Na^+ and K^+ are even steeper than normal and that the ratio [ATP]/[ADP] [P_i] is lower than normal, we can make the pump run backwards and synthesize ATP at the expense of downhill movements of the cations (Garrahan & Glynn 1967*d*). We have not been able to obtain accurate figures for the stoichiometry of the pump when it is running backwards, but the synthesis of each molecule of ATP is associated with the loss of something like two or three potassium ions (Glynn & Lew 1970). The number of sodium ions entering the cell for each molecule of ATP synthesized is much more than three, but this is because most of the ouabain-sensitive Na^+ entry is associated with the rapid Na–Na exchange that occurs when red cells are incubated in media rich in Na^+ and lacking K^+ (Garrahan & Glynn 1967*a*).

This Na–Na exchange, which has also been seen in giant axons (Baker *et al.* 1969; De Weer 1970) and probably also in frog muscle (Keynes & Steinhardt 1968; Horowicz *et al.* 1970; Sjodin 1971), is roughly one-for-one, but it shows a marked asymmetry in the affinity for Na^+ on the two sides of the membrane: the affinity is high at the inner surface and extremely low at the outer (Garrahan & Glynn 1967*a*; Garay & Garrahan 1973). The exchange of sodium ions is associated with the hydrolysis of little or no ATP (Garrahan & Glynn 1967*c*), but experiments on resealed ghosts containing a regenerating system to control the concentrations of ATP and ADP have shown that the exchange varies linearly with the concentration of ADP up to 300 μmol/l, the highest concentration tested (Glynn & Hoffman 1971). A dependence on ADP has also been demonstrated in squid axons (De Weer 1970). In red cells, changes in ATP concentration in the range 300–1500 μmol/l have little effect. We suspect that ATP in low concentrations is required, but the presence of adenylate kinase makes it impossible to prepare ghosts containing ADP without ATP, so a requirement for ATP cannot be tested.

The fact that ADP is not required for the normal working of the pump but is required for Na–Na exchange suggests that the entry of Na^+ is somehow

associated with the rephosphorylation of ADP by the phosphorylated pump protein. The inward movement of Na^+ must, however, require a step in addition to the transfer of phosphate, since oligomycin blocks the Na–Na exchange (Garrahan & Glynn 1967*c*) but stimulates the Na^+-dependent ATP–ADP exchange which is thought to reflect the transfer of phosphate to and from the enzyme (Blostein 1968, 1970).

The K–K exchange proceeds to some extent under physiological conditions (Glynn *et al.* 1970), but it is best studied in resealed ghosts containing little Na^+. Like Na–Na exchange it is roughly one-for-one and is not associated with the hydrolysis of ATP (Simons 1974). The apparent affinities for K^+ on the two sides of the membrane are strikingly asymmetric, with a high affinity outside and a low affinity inside (Glynn *et al.* 1970; Simons 1974). This behaviour is just the opposite of that in Na–Na exchange and raises the possibility that the same sites participate in both exchanges. An important difference between K–K exchange and Na–Na exchange is that the exchange of K^+ does not require ADP (Simons 1974) but does require the presence of inorganic phosphate at the inner surface of the cell membrane (Glynn *et al.* 1970). If phosphate is reversibly transferred, it must therefore be transferred between phosphoenzyme and water rather than between phosphoenzyme and ADP. Such a transfer would lead to the exchange of oxygen atoms between water and inorganic phosphate, and an exchange of this kind, which is stimulated by K^+, has recently been described (Dahms & Boyer 1973) in experiments with fragmented membranes.

Remarkably, potassium ions are not exchanged in cells lacking nucleotide (Glynn *et al.* 1971) but, recently, T. J. B. Simons (unpublished results) has shown that the non-phosphorylating β,γ-imido or methylene analogues of ATP can replace ATP. Whatever the role of ATP may be, it cannot include phosphorylation.

The fifth mode, the uncoupled Na^+ efflux, is seen when red cells or resealed ghosts are incubated in choline or Mg^{2+} media (Garrahan & Glynn 1967*a*,*b*; Lew *et al.* 1973; Karlish & Glynn 1974; Beaugé & Ortiz 1973). A similar uncoupled efflux has been described in crab nerves (Baker 1964) and frog muscle (Beaugé & Ortiz 1972), but it is only in red cells that we can be sure the efflux does not represent an exchange with potassium ions that have leaked from the cells.

The uncoupled efflux involves the combination of intracellular sodium ions with sites of fairly high affinity on the inner surface of the membrane (Karlish & Glynn 1974, but cf. Eilam & Stein 1973), and the efflux is associated with the hydrolysis of ATP—about three sodium ions leave for each molecule of ATP hydrolysed (Glynn & Karlish, unpublished work). We suspect that the un-

coupled Na efflux is related to the ATPase activity seen with fragmented membranes at low ATP concentrations when Na^+ is present without K^+. It is striking that nucleoside triphosphates which bind to the pump much less strongly than ATP are better at replacing ATP as a substrate for Na^+-activated hydrolysis than as a substrate for ($Na^+ + K^+$)-activated hydrolysis (Tobin *et al.* 1972; Skou & Hilberg 1969; Siegel & Goodwin 1972). Similarly, they are better at replacing ATP as a fuel for the uncoupled Na^+ efflux than as a fuel for Na–K exchange (Karlish & Glynn 1974). With low enough concentrations of poorly bound nucleotides, K^+ inhibits both hydrolysis and Na^+ efflux. A curious feature of the uncoupled Na^+ efflux that is not yet understood is its strong inhibition by low concentrations of Na^+ in the medium (Garrahan & Glynn 1967*a*,*b*; Karlish & Glynn 1974).

The picture that emerges from the study of all these abnormal fluxes and the biochemical changes accompanying them is that: (i) the outward movement of Na^+ is associated with a transfer of phosphate from ATP to the enzyme; (ii) the inward movement of Na^+ is associated with the transfer of phosphate from the phosphoenzyme to ADP; (iii) the inward movement of K^+ is associated with the transfer of phosphate from the phosphoenzyme to water; and (iv) the outward movement of K^+ is associated with phosphorylation of the enzyme by inorganic phosphate. By listing these associations separately, we do not mean to imply that the different steps necessarily occur independently (see later). Once the phosphoenzyme has been formed its fate depends on the composition of the external medium: if K^+ is present, phosphate is transferred to water and K^+ enters; if Na^+ is present and K^+ is not, the phosphoenzyme transfers its phosphate to ADP and Na^+ enters; if neither Na^+ nor K^+ is present in the external medium, then the phosphate is transferred slowly to water, and the slow overall hydrolysis of ATP is accompanied by a slow uncoupled efflux of Na^+.

Until recently it was customary to explain these associations of fluxes and biochemical changes by some kind of circulating carrier model, like those discussed by Shaw (1954) and Caldwell (1969), in which a carrier responsible for moving Na^+ outwards is converted at the outer face of the membrane into a carrier moving K^+ inwards. Energy is supposed to be fed into the system by driving the conversion of one form of the carrier into the other at one face of the membrane, the reversion at the opposite face occurring spontaneously.

Circulating carrier models provide a ready explanation of the coupling between Na^+ and K^+ movements, and they can account for the various abnormal fluxes and for the associated biochemical changes. But they have had to be abandoned for two quite different reasons.

First, in the circulating carrier model, the apparent affinities for the ions at

each surface of the membrane are complicated functions of the rate constants of the various reactions in the cycle. Except by chance, they are not equal to the true binding constants, and each depends on the ion concentrations at the opposite surface. Some years ago, Baker & Stone (1966) pointed out that the dependence of the apparent affinity for ions at each surface on the conditions at the opposite surface, provided a criterion by which circulating carrier models could be tested. The experiments are technically troublesome, but in the last few years Hoffman & Tosteson (1971) and Garrahan & Garay (1974) have applied this criterion. In both cases it is clear that the apparent affinities at each surface of the membrane are independent of the nature and concentration of the alkali-metal ions at the opposite surface. The relation between flux and cationic concentrations can be described by the product of a constant and two factors which express the saturation of the activation sites at the internal and external surfaces.

The simplest interpretation of these findings is to suppose that sodium and potassium ions may bind rapidly, randomly and independently at the internal and external sites, and that transport proceeds only when the pump has bound both Na^+ and K^+. If this is correct, the internal Na^+ sites and the external K^+ sites must exist simultaneously and not merely consecutively.

Independent evidence for the simultaneous existence of internal Na^+ sites and external K^+ sites comes from work with irreversible inhibitors (Robinson 1973, 1974*a*,*b*). If the rate of onset of inhibition by an irreversible inhibitor is affected by the concentration of a ligand, then measurement of the rate of inactivation at different ligand concentrations gives a measure of the affinity of the ligand for the site at which it exerts its effect on the inhibition. If that happens to be the site at which the ligand also has physiological effects, interesting information can be obtained.

Both beryllium ions and fluoride ions inhibit the Na^+,K^+-ATPase, provided that both magnesium and potassium ions are present. The site at which potassium ions promote inhibition seems to be the same as that at which they act to promote hydrolysis of ATP, or hydrolysis of the so-called 'phosphatase substrates'—substances like *p*-nitrophenyl phosphate or acetyl phosphate, which preparations of Na^+,K^+-ATPase hydrolyse in the presence of K^+. The evidence for this identity of sites is, first, that the relative effectiveness of K^+, Tl^+ and NH_4^+ is the same for the different processes, secondly, that the K^+ affinity for the different processes is similarly affected by phlorizin, and, thirdly, that there is a striking parallelism between the effects of ATP plus Na^+ or CTP plus Na^+ on the sensitivity to K^+ of (i) the rate of onset of inhibition by beryllium and (ii) the rate of hydrolysis of phosphatase substrates (Robinson 1973). The promotion of ATP hydrolysis, or the hydrolysis of the

'phosphatase substrates', by K^+ is known to be an effect of K^+ acting at external sites (Rega *et al.* 1970), and the rate of onset of inhibition by beryllium or fluoride ions at different concentrations of K^+ can therefore be used to gain information about the affinity of these sites.

Robinson (1973,1974*a*) has used this approach to measure the affinity of the external K^+ sites in different conditions. On the circulating carrier model, K^+ sites should not exist until after phosphorylation had occurred and Na^+ had been moved outwards. What Robinson found was that, even in the absence of ATP, external sites existed with a moderately high affinity for K^+ ($K_{0.5}$ = 1.4 mmol/l when $[Na]_0 = 0$). When Na^+ plus ATP or Na^+ plus CTP were present, phosphorylation occurred, and sites with an affinity for K^+ more than ten times as great were detectable. Clearly, the appearance of high affinity sites depended on phosphorylation, since Na^+ plus EDTA, or Na^+ plus the non-phosphorylating β,γ-methylene analogue of ATP, were ineffective. These results prove that sites of moderately high affinity for K^+ exist before phosphorylation and they strongly suggest that phosphorylation greatly increases the affinity of these sites. It is also reasonable to assume that the well known effect of Na^+ plus ATP, or Na^+ plus CTP, in making the hydrolysis of 'phosphatase substrates' more sensitive to K^+ (Nagai & Yoshida 1966) is the result of phosphorylation of the enzyme and a consequent increase in K^+ affinity of the sites at which K^+ activates.

Robinson (1974*b*) was also interested to discover whether phosphorylation affects the affinity of the inward-facing Na^+ sites. Sodium ions can protect against inhibition by dicyclohexylcarbodiimide, and Robinson showed that the sites at which Na^+ exerts its protective action must be the internal Na activation sites, because the $K_{0.5}$ for protection by Na^+ in the absence of other ligands was the same as the $K_{0.5}$ for Na^+ activation of the ATPase at low K^+ concentrations, and the $K_{0.5}$ for both effects was altered similarly by phlorizin. The interesting conclusion from Robinson's work is that phosphorylation, which, as we have seen, appears to cause a large increase in the affinity of the external K^+ sites, has little effect on the affinity of the internal Na^+ sites.

PHOSPHORYLATED INTERMEDIATES

When Na^+,K^+-ATPase preparations are exposed to [γ-^{32}P]ATP in the presence of Mg^{2+} and Na^+, the enzyme is phosphorylated; if K^+ is then added the ^{32}P is released as inorganic phosphate. There has been a good deal of controversy about whether the phosphoenzyme is an intermediate in the normal course of events, when K^+ is present all the time, though evidence from studies

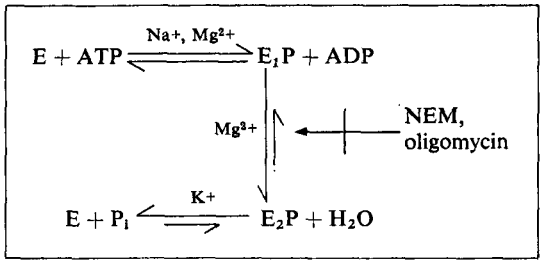

FIG. 2. A scheme to explain the different properties of phosphoenzyme formed in different conditions (modified from Siegel & Albers 1967): NEM, *N*-ethylmaleimide.

of the steady state (Kanazawa *et al.* 1967; Neufeld & Levy 1970) and from experiments with rapid-mixing techniques (Kanazawa *et al.* 1970; Mårdh & Zetterqvist 1972, 1975; Mårdh 1974) makes it likely that the phosphoenzyme is the normal intermediate.

Another controversy concerns the nature of different forms of the phosphoenzyme and the sequence in which they are formed. It is generally agreed that the phosphoenzyme formed by phosphorylation in the presence of Na^+ and at normal Mg^{2+} concentrations can be rapidly hydrolysed in the presence of K^+ but is not much affected by ADP. If the enzyme is phosphorylated in the presence of oligomycin or very low Mg^{2+} concentrations, or if it has been pretreated with *N*-ethylmaleimide, then the phosphoenzyme formed is susceptible to ADP but not to K^+ (Fahn *et al.* 1966*a*,*b*; Post *et al.* 1969). The widely accepted interpretation of these findings (Siegel & Albers 1967) (see Fig. 2) is that the phosphoenzyme first formed, E_1P, is converted into a form of lower energy, E_2P, by a reaction that is not readily reversible, that requires high Mg^{2+} concentrations and that is blocked by oligomycin or *N*-ethylmaleimide. Tonomura and his colleagues have criticized this hypothesis on the basis of four kinds of experiment (Kanazawa *et al.* 1970; Fukushima & Tonomura 1973).

The rate constant for the breakdown of the phosphoenzyme can be estimated *either* by measuring the rate constant, k_D, of the disappearance of ^{32}P-labelled phosphoenzyme when its synthesis is suddenly stopped by the addition of EDTA or an excess of unlabelled ATP, *or* by dividing the steady-state rate of hydrolysis of ATP, v, by the concentration of phosphoenzyme ([EP]) present in the steady-state (i.e. $v/[EP]$). In the absence of K^+, the two methods gave the same answer, but in the presence of K^+ the ratio $v/[EP]$ exceeded k_D and was almost double k_D when $[K^+]$ was 0.6 mmol/l. In the second kind of experiment, Tonomura and his colleagues suddenly stopped the synthesis of labelled phosphoenzyme, by the addition of EDTA or an excess of unlabelled

ATP, and then compared the rate of disappearance of labelled phosphoenzyme with the rate of appearance of labelled inorganic phosphate. Again the two rates agreed well in the absence of K^+, but with 0.6mM-K^+ the rate of appearance of P_i was about twice the rate of disappearance of ^{32}P from the phosphoenzyme.

To explain both these discrepancies they proposed that ATP must have been bound to the enzyme in such a way that it could be released by trichloroacetic acid but could continue to form phosphoenzyme after the addition of EDTA or the excess of unlabelled ATP. They supposed that in the presence of 0.6mM-K^+ the equilibrium between phosphoenzyme and the form of the enzyme binding ATP was about evenly poised, whereas in the absence of K^+ the equilibrium greatly favoured the phosphoenzyme.

In support of this suggestion, they showed that if K^+ and EDTA were added to enzyme that had just been phosphorylated, the concentration of the phosphoenzyme fell rapidly at first before a slower exponential loss, and the rapid fall was accompanied by the appearance of an equivalent amount of ATP. Finally, in experiments on enzyme pretreated with *N*-ethylmaleimide, they showed that newly formed phosphoenzyme was not sensitive to ADP but became sensitive within a second or two at 0 °C. They supposed that this newly formed phosphoenzyme did not react with added ADP because it still had ADP bound to it. Hence, they suggested that the sequence in Albers' scheme was wrong and that the ADP-insensitive form was formed first.

This hypothesis of Tonomura and his colleagues is difficult to reconcile with the results of an experiment by Post *et al.* (1969). They incubated membrane fragments with Mg^{2+} and [γ-^{32}P]ATP in the absence of Na^+; the ATP bound to the enzyme but did not phosphorylate it. They then added a 'chasing' solution containing Na^+ and unlabelled ATP. The Na^+ allowed the labelled ATP bound to the enzyme to phosphorylate, so that the amount of membrane-bound ^{32}P rose rapidly and then slowly fell as the phosphoenzyme broke down. The crux of the experiment was that when ADP was present in the chasing solution, the initial rise in membrane-bound ^{32}P was smaller but the rate constant for its disappearance was unaltered. This strongly suggests that the ADP-sensitive form of the phosphoenzyme precedes the ADP-insensitive form.

To accommodate these findings and those of Tonomura and his colleagues, it seems to be necessary to suppose that the phosphoenzyme goes through three states (Fig. 3). The first-formed state is insensitive to added ADP because it still has ADP bound to it. The bound ADP then dissociates to leave an ADP-sensitive form, $E_1 \sim P$, which is transformed into an ADP-insensitive, K^+-sensitive form, E_2P.

An interconversion between ADP-insensitive and ADP-sensitive forms is necessary to explain the experimental results. The part of the scheme in Fig. 3

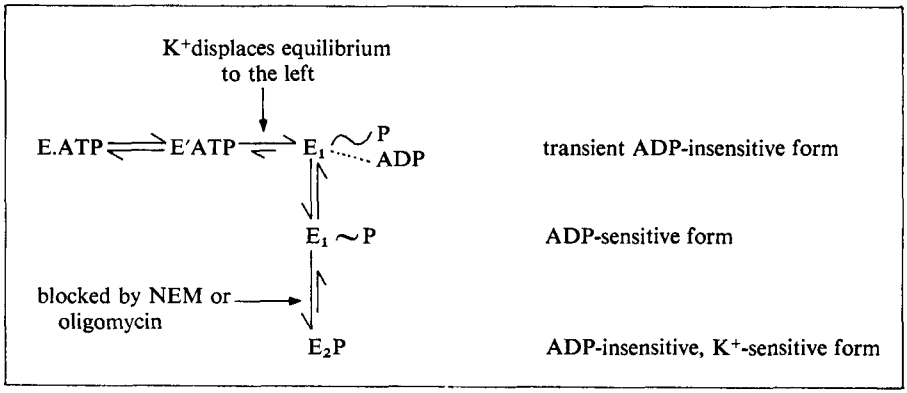

FIG. 3. A scheme to reconcile the findings of Kanazawa *et al.* (1970) with the reaction sequence suggested by Siegel & Albers (1967). An alternative, and perhaps more economical, interpretation of the results of Kanazawa *et al.* (1970), assuming that the Na^+,K^+-ATPase shows half-of-the-sites reactivity, is given below.

that looks arbitrary, is the K^+-dependent equilibrium between phosphoenzyme and the form of the enzyme with bound ATP (E'ATP) that Tonomura and his colleagues postulated to explain the discrepancies between $v/[EP]$ and k_D and between the rate of loss of the phosphoenzyme and the rate of formation of P_i. We can make this part of the scheme much more attractive if, following the original suggestion of Stein *et al.* (1973), we suppose that the Na pump shows half-of-the-sites reactivity, like the alkaline phosphatase from *E. coli* (see Lazdunski 1972). That enzyme is a dimer and the characteristic feature of its behaviour is that, because of conformational changes associated with phosphorylation or binding of substrate, only one subunit can bind phosphate covalently or ATP non-covalently at any time. The phosphorylation of one subunit is accompanied by dephosphorylation of the other, so that the two subunits go through the reaction cycle out of phase. If the Na pump behaves analogously, we can imagine that the tightly bound [γ-^{32}P]ATP that continued to phosphorylate in Tonomura's experiments after the reaction with [γ-^{32}P]ATP in the medium had been blocked was simply ATP bound to the other subunit of the pump, that is ATP in the form $E_1 \cdot ATP–E_2P$. In the absence of K^+, Tonomura and his colleagues found no discrepancy between $v/[EP]$ and k_D or between the rate of loss of the phosphoenzyme and the rate of formation of P_i. This could be *either* because the lower rate of dephosphorylation allowed ATP to dissociate from the E_1 part of the enzyme before the breakdown of E_2P, *or* because, in the abnormal situation of the absence of K^+, both halves of the enzyme were phosphorylated. This is a reasonable suggestion because Albers *et al.* (1968) found that when fragments of electric organ membrane

were exposed to Na^+ and [γ-^{32}P]ATP, two atoms of ^{32}P were incorporated per ouabain binding site (although in similar experiments with cat brain membranes only one ^{32}P was incorporated per ouabain binding site). More recently, Jørgensen (1974) reported that a purified Na^+,K^+-ATPase preparation from rabbit kidney contained two phosphorylation sites per ouabain binding site. The idea that the pump is a dimer is also supported by structural studies (Kyte 1971; Lane *et al.* 1973; Jørgensen 1974; but see also Kyte 1972; Hokin *et al.* 1973; Hokin 1975), and by data on ATP binding (Hegyvary & Post 1971), on ouabain binding (Taniguchi & Iida 1972) and on radiation inactivation of different partial reactions of the pump (Kepner & Macey 1968; J. C. Ellory & S. R. Levinson, unpublished experiments).

It is crucial to the kind of explanation we suggest, not only that the pump is a dimer, but that forms of it exist with $\sim$P bound to one half and ATP bound to the other half. The recent finding (Tobin *et al.* 1974) that non-phosphorylating ATP analogues release ouabain from phosphoenzyme provides some support for this view.

The fact that the phosphoenzyme is sensitive to ADP only in special conditions can also be explained simply on the half-sites-reactive scheme if we suppose that the binding of ATP to the E_2 component of the form E_1(ADP)P–E_2 leads to the release of ADP and the immediate formation of E_2P–E_1 ATP. Conditions which lead to ADP-sensitivity are presumably those in which ADP is released from the enzyme without the change in conformation.

When fragmented membranes are incubated at 37 °C in the presence of Na^+ and K^+, ADP competes with ATP but does not act as a non-competitive product inhibitor (Hexum *et al.* 1970). This result suggests that the ADP-sensitive form of the phosphoenzyme has only a transient existence in these conditions.

Equivalence of the two halves of the dimer

If the pump is a dimer which behaves as a half-sites enzyme, we can ask whether, in the absence of substrates and physiological ligands, the two halves are equivalent (E–E) or not (E_1–E_2). If they are not equivalent we might expect only one half to be capable of catalysing reactions such as the hydrolysis of *p*-nitrophenyl phosphate or ouabain binding; and to inhibit these partial reactions by irradiation it would, therefore, only be necessary to damage the relevant half. If the two halves are equivalent, inhibition of the partial reactions would require inactivation of both halves of the enzyme, even though, presumably, only one half can act at a time. The two hypotheses lead to different predictions of the relative target sizes when irradiation is used to knock out

Na^+,K^+-ATPase activity (involving the whole enzyme) or partial reactions supposedly involving only one monomer.

If A_0 is the total number of enzyme molecules in 1 cm^3, A is the number of enzyme molecules in 1 cm^3 remaining intact after time t, $2V$ is the volume of the dimer in cm^3 and D is the dose rate in (inactivating events)/cm^3 s, then the rate of loss of enzyme is given by equation (1), which on integration gives equation (2).

$$\frac{dA}{dt} = -2VDA \tag{1}$$

$$\frac{A}{A_0} = e^{-2VDt} \tag{2}$$

For 37% (i.e. 1/e) survival of ATPase activity, $2VDt = 1$. If the enzyme is E_1–E_2 and only E_2 can catalyse, say, the hydrolysis of *p*-nitrophenyl phosphate (*p*NPP), then a similar argument shows that for 37% survival of *p*NPPase activity $VDt = 1$.

If the enzyme is E–E, both halves must be inactivated to prevent *p*NPPase activity. The fraction of enzyme with both halves inactivated will be $(1 - e^{-VDt})^2$, and the fraction of *p*NPPase activity remaining will therefore be $1 - (1 - e^{-VDt})^2$. The dose effect curve will not be linear on a semilogarithmic plot, and for 37% survival of *p*NPPase activity $VDt = 1/1.77$.

If the apparent molecular weight is calculated from the dose required for 37% survival, assuming one-hit inactivation, it follows that the ratio (apparent mol.wt. of ATPase)/(apparent mol.wt. of *p*NPPase) should be 2:1 if the enzyme is E_1–E_2, and 3.5:1 if the enzyme is E–E. Accurate figures for these ratios are not available, but the results of Kepner & Macey (1968), who looked at the effects of irradiation on ATPase and *p*NPPase activity, and of J. C. Ellory & S. R. Levinson (unpublished results), who looked at the effects of irradiation on ATPase and *p*NPPase activity and on ouabain binding, are compatible with a ratio of 2:1 but not with a ratio of 3.5:1. The tentative conclusion is, therefore, that even in the absence of substrates and physiological ligands the two halves of the enzyme are in different states. If this conclusion is correct, the pump is different from the classical half-sites enzyme in which differences between the two subunits are induced only by the binding of substrates or by reactions that follow substrate binding.

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Discussion

Keynes: If the old circulating-carrier model is now so unfashionable, what alternative do you propose?

Glynn: I don't know. We can account for the independence of the affinities (for Na^+ and K^+ on each side of the membrane) of the conditions on the opposite side if we suppose that two ion-transporters, or carriers, are coupled back-to-back so that Na^+-carriers and K^+-carriers exist simultaneously. A particularly interesting question is whether the ions are transported across the membrane in one move or whether there is an interchange in an occluded region of the membrane. Stein *et al.* (1973) suggested an internal exchange of this kind, largely to explain a feature of the uncoupled Na^+ efflux, namely its inhibition by low Na^+ concentrations outside the cell. The sensitivity of the uncoupled Na^+ efflux to external Na^+ points to the existence of a high-affinity Na^+ site on the outside surface of the cell, different from the low-affinity Na^+ sites involved in the Na–Na exchange. Stein's hypothetical mechanism provides a neat explanation for the existence of this external high-affinity site, but whether the explanation is true as well as ingenious I don't know.

Klingenberg: In the proposed dimer model (p. 214) with two different subunits, would both be biochemically identifiable? Can you identify one subunit which binds ouabain from the other which binds ATP? Erdmann & Schoner

(1973) elegantly showed that ouabain can bind only from the outside and then prevents binding of ATP from the inside.

Glynn: The two subunits in each half of the dimer differ greatly—their molecular weights are disparate and only the large subunit contains the phosphorylation site. I don't know which binds ouabain. To distinguish the biochemical properties of the corresponding subunits in the two halves of the dimer is more difficult. The fact that in the absence of K^+ and Mg^{2+} the ATPase binds only one molecule of ATP presumably implies that the ATP-binding site in the other half of the dimer is inaccessible. Taniguchi & Iida (1972) showed that the ATPase can bind two molecules of ouabain, one with a high affinity and one with a much lower affinity, and this fact suggests that the properties of the ouabain-binding sites differ in the two halves of the dimer. Incidentally, the existence of a second ouabain-binding site, with a low affinity, explains a puzzle that has been worrying people for a long time: in measuring ouabain-binding sites associated with sodium pumps, many people have reported considerable 'unspecific' binding which can be avoided by the use of either K^+ or Cs^+. It has always seemed suspicious that one of the ligands handled by the pump happens to prevent unspecific binding. Now, it seems likely that it is binding at the second site that is prevented by K^+ or Cs^+.

Lipmann: You gave the impression of two phosphate bindings of a different type: one with carboxy groups to form an energy-rich bond, the other possibly of an energy-poor type. Do you think that the phosphate can switch on the enzyme between two sites?

Glynn: I think not. By phosphorylating the ATPase with ATP or inorganic phosphate in various conditions one can get phosphoenzyme which is *either* sensitive to K^+ but not to ADP *or* sensitive to ADP but not to K^+, but the electrophoretic patterns of the fragments of proteolytic digestion of the phosphoenzyme formed in the various different ways are indistinguishable. Since the chemistry is common, the differences presumably lie in the configuration.

Lipmann: Since the energy needed to put inorganic phosphate into a carboxy phosphate is considerable, one feels somewhat uneasy about making the phosphate go to a carboxy group.

Glynn: Is it inconceivable to have an acyl phosphate that is a relatively low-energy compound while it is *in situ* in the lipid membrane?

Lipmann: That is possible if the enzyme assumes a new conformation. But then if one wants the phosphoryl to return to ADP, one has, in some way, to switch back to a high-energy bond.

Glynn: The formation of phosphoenzyme (with the phosphate attached to an acyl group) from inorganic phosphate was first reported after experiments with ouabain (Lindenmeyer *et al.* 1968; Albers *et al.* 1968). At that time it

was assumed that ouabain stabilized the acyl phosphate. Then, Post *et al.* (1973) discovered that some phosphorylation by inorganic phosphate took place even in the absence of ouabain. Phosphoenzyme so formed must presumably have the phosphate in a fairly low-energy form. To transfer the phosphate from that form to ADP one must supply energy either from a concentration gradient, as in our experiments on resealed ghosts, or by suddenly changing the composition of the medium, as Post *et al.* (1974) recently did. They showed that membrane preparations treated with inorganic phosphate in the absence of both Na^+ and K^+ formed a small amount of phosphoenzyme that was peculiar in being insensitive both to K^+ and ADP. When a large amount of EDTA and Na^+ in heroic concentrations were added, the phosphoenzyme was slowly converted into a form which was able to phosphorylate ADP. Since the preparation used by Post *et al.* was vesicular, we do not know whether the introduction of energy involved the setting up of a Na^+ concentration gradient across the membrane of the vesicle.

Huxley: Is this question of the possible existence of an acyl phosphate of lower energy related to the matter of bound ATP being formed from ADP and P_i on myosin (Gutfreund & Trentham, pp. 69–81)? The latter is an example of a nominally high-energy bond which is nevertheless capable of being formed from P_i in a bound state on the enzyme.

Gutfreund: It need not be a phosphoryl enzyme to reverse the reaction.

Lipmann: I might mention here the case of alkaline phosphatase which can bind P_i to the hydroxy group of serine. This is stabilized only in special conditions: the pH must be such as to suppress hydrolysis (i.e. an acidic solution). The carboxy phosphate impresses as being rather energy-rich—I don't want to dispute that—yet the phosphate can be transferred to the carboxy group from a specially-located seryl hydroxy group.

Gutfreund: Although the subunit model is interesting, there is no advantage in making analogies with half-site reactivity in enzymes. Not only is it in any case different, but the enzyme model is based on shaky ground—especially, you should not talk about the classical half-site enzyme.

If your protein is a device that works in a reciprocating manner, this could be a 'flip-flop' mechanism. A 'flip-flop' device has a memory: it is misleading to apply their analogy to mechanisms which are not understood in detail (Gutfreund 1975).

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The bacterial phosphoenolpyruvate : sugar phosphotransferase system

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Abstract The bacterial phosphotransferase system participates in diverse physiological phenomena; its best characterized function is in the group translocation of sugars that are substrates of the system. Such sugars are phosphorylated as they are translocated across the cell membrane. Isolation of different proteins of the phosphotransferase system and reconstitution of the complex shows that in the net transfer of the phosphoryl group from phosphoenolpyruvate to a given sugar the phosphoryl group is sequentially transferred from one protein to another. In all cases so far studied, with one important exception, the phosphoryl group is linked to the proteins through a nitrogen atom in the imidazole ring of a histidyl residue. In the exceptional protein, the phosphoryl group is linked to a carboxy group.

An additional function of the phosphotransferase system is to regulate the uptake of sugars that cannot be phosphorylated.

An obligatory requirement for cell survival is that membranes must discriminate between similar solutes, such as sodium and potassium ions, or between D-glucose and L-glucose. The molecular mechanisms underlying selective permeability are not known, despite an exponential increase in the rate of work on cell membrane structure and function over the past decade. In this paper, I shall describe an energy-using system responsible for the translocation of several carbohydrates across bacterial cell membranes.

The bacterial phosphotransferase system (PTS), accidentally discovered in 1964 (Kundig *et al.* 1964), was shown to phosphorylate several carbohydrates. The system exhibited two unique features: its complexity and its phosphoryl donor, phosphoenolpyruvate. Three protein fractions take part in the overall reaction. One protein, designated HPr, is relatively stable to heat; of the other two, Enzymes I and II, the former catalyses the transfer of the phosphoryl group from phosphoenolpyruvate to HPr and the latter transfers the group

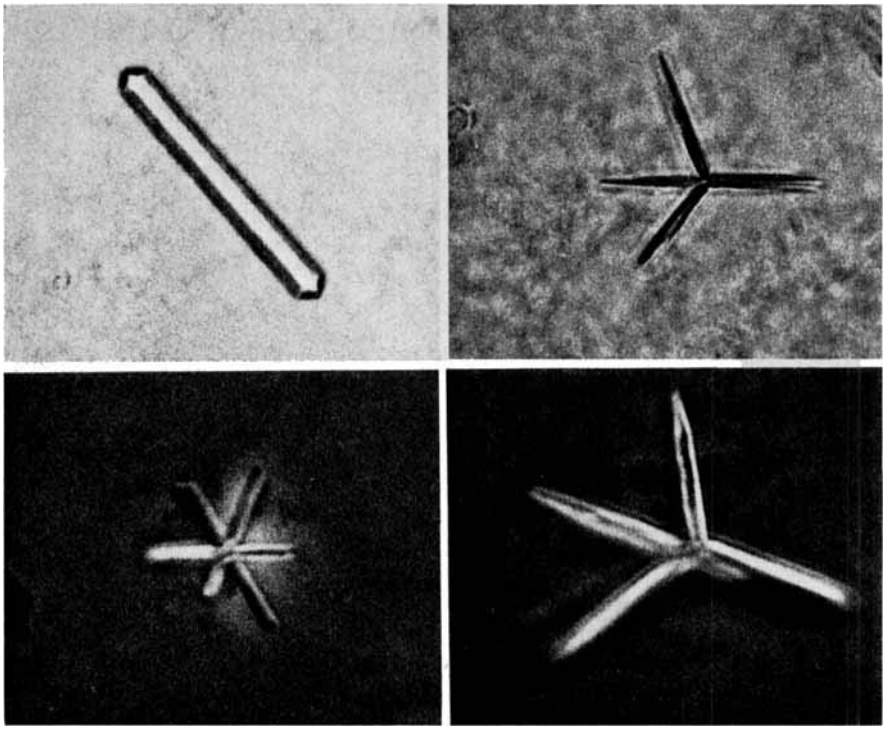

FIG. 1. Photomicrographs of HPr crystals.

from phosphoHPr to the sugars. The phosphoryl group in phosphoHPr is linked to a nitrogen atom in the imidazole ring of a histidyl residue.

In a series of studies aimed at further defining the enzyme system, the protein fractions were purified and HPr was crystallized (Fig. 1). The overall reaction consists of a sequential transfer of the phosphoryl group from phosphoenolpyruvate through a chain of proteins to the sugar (as depicted in Figs. 2 and 3). The first two proteins in the system, Enzyme I and HPr are called general proteins of the phosphotransferase system in the sense that they are not sugar-specific but are required for the phosphorylation of all sugars that are substrates of the system (the PTS sugars). The catalytic steps beyond P~HPr require the participation of sugar-specific proteins. In every case we have studied in detail, the transfer of the phosphoryl group from phosphoHPr to a sugar requires a pair of proteins specific for that sugar or a duplex of sugar-specific proteins. A few duplexes are found regardless of the carbon source used for cell growth (i.e., constitutive), whereas other sugar-specific duplexes are detected by growing the cell on the relevant sugar (i.e., inducible). How many

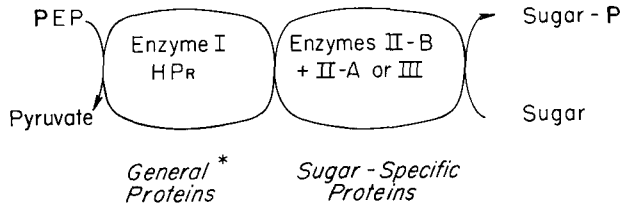

FIG. 2. The phosphotransferase system. The overall reaction from phosphoenolpyruvate and sugar to phosphosugar and pyruvate requires four proteins; two are general proteins (*, not sugar specific) and two sugar-specific.

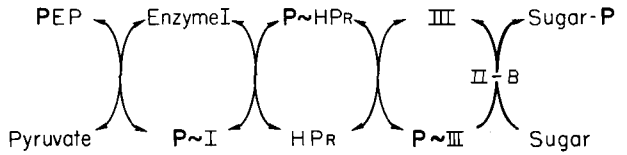

FIG. 3. Transfer of the phosphoryl group in the phosphotransferase system. The phosphoryl group is transferred sequentially from phosphoenolpyruvate (PEP) to the first general protein, Enzyme I, to the second general protein, HPr, to one of the sugar-specific proteins (III) and finally to the sugar. The last step requires the second sugar-specific protein (II-B).

duplexes can be generated by a particular cell type is not yet known. With highly purified or homogeneous proteins it has been possible to show that the phosphoryl group is transferred from phosphoHPr to one of the sugar-specific proteins and finally to the sugar through the intermediacy of the second sugar-specific protein of the duplex (Fig. 3). Further, in the cases tested so far, the last protein in Fig. 3 (II-B) is the sugar-binding or sugar-receptor protein.

GENERAL PROTEINS

Enzyme I, a soluble protein, has been purified to apparent homogeneity, but has not yet been properly characterized by physicochemical techniques. The purified protein is extremely labile, sensitive to reagents for thiols and contains an unknown number of subunits (possibly not identical) with a molecular weight of about 80 000 daltons (determined by gel filtration chromatography). In the phosphoenzyme, the phosphoryl group is attached to histidine. Mutants defective in Enzyme I are grossly affected not only in their ability to take up PTS sugars from the medium but in many more complex phenomena, such as catabolite repression. A detailed analysis of the protein may, therefore, reveal that it contains regulatory as well as catalytic subunits, and an understanding

of how it is regulated may shed light on the apparent essentiality of this protein in normal bacterial physiology.

The protein HPr has been purified to homogeneity from *Escherichia coli* (Anderson *et al.* 1971), from *Salmonella typhimurium* and from *Staphylococcus aureus* (Simoni *et al.* 1973*a*). The HPr proteins from *E. coli* and *S. typhimurium* appear to be identical (with molecular weight 9500), but differ from the protein isolated from *S. aureus* (molecular weight 8600). Furthermore, the *E. coli* protein contains two mol of histidine but no tyrosine, tryptophan or cysteine, whereas *S. aureus* HPr contains one mol of histidine, two mol of tyrosine but no tryptophan or cysteine. The two types of HPr react at negligible rates when substituted for each other in the heterologous phosphotransferase systems. Derivatives of active HPr proteins as well as two mutant forms of the protein have been prepared. We hope that some derivatives, such as the dansylated protein, will help us in elucidating the enzymic phosphotransfer reactions and the mechanism of sugar translocation by the PTS. The complete amino acid sequence of HPr from *S. typhimurium* is being analysed and Dr W. Love (Dept. of Biophysics, Johns Hopkins University) is trying to obtain crystals suitable for X-ray work.

In the phosphorylated HPr from *E. coli*, which has been isolated (Anderson *et al.* 1971), the phosphoryl group is linked at the N-1 position of the imidazole ring of a histidyl residue, a linkage even more labile than that in N^3-phosphohistidine (Hultquist 1968). PhosphoHPr is the second phosphohistidyl protein isolated, the first being the phosphorylated form of succinyl-CoA synthetase (Boyer *et al.* 1962), in which the phosphoryl group is linked at the N-3 position of the imidazole ring.

The apparent equilibrium constant for reaction (1) has been determined

$$\text{Phosphoenolpyruvate} + \text{HPr} \underset{\text{I}}{\leftrightharpoons} \text{PhosphoHPr} + \text{Pyruvate} \qquad (1)$$

to be 10 ± 5 (Simoni *et al.* 1973*b*). From this result, the apparent standard free energy of hydrolysis of phosphoHPr is calculated to be −56.1 kJ/mol (−13.4 kcal/mol). This value shows that the N-1 linkage is truly of the 'high energy' type; it is somewhat less than that of phosphoenolpyruvate and about twice that of the pyrophosphate bonds in ATP.

SUGAR-SPECIFIC PROTEINS

Table 1 summarizes those sugar-specific duplexes for which at least one member of the duplex has been isolated in apparently homogeneous form.

A point of especial importance to the physiological role or roles of the phosphotransferase system is that we have invariably found that at least one

TABLE 1

Sugar-specific duplexes of the phosphotransferase system. Proteins derived from exhaustively washed membranes are designated II; soluble proteins are called III.

System	*Sugar*	*Proteins*	
		Membrane	*Soluble*
II-A, II-B			
E. coli (constitutive)	Glucose	II-A^{Glc}, II-B	
	Fructose	II-A^{Fru}, II-B	
	Mannose	II-A^{Man}, II-B	
III, II-B			
E. coli, constitutive	Glucose	II-B^{Glc}	III^{Glc}
S. aureus, inducible	Lactose	II-B^{Lac}	III^{Lac}

member of each duplex is a tightly-bound membrane component; these proteins are designated II-B. The other member of a given duplex may be found in the supernatant fluid of cell-free homogenates (III) or may be a membrane component (II-A). (The cumbersome nomenclature in use for these proteins requires a subscript to indicate the organism from which the proteins were isolated—Ec, *E. coli*; St, *S. typhimurium*; Sa, *S. aureus*—and a superscript to indicate sugar specificity, for example, $III^{Lac}_{Sa}/II\text{-}B^{Lac}_{Sa}$ is the lactose-specific duplex isolated from *S. aureus*.)

The constitutive duplexes in membranes of *E. coli* and of *S. typhimurium* have been studied (Kundig & Roseman 1971). Exhaustively washed membranes from these cells phosphorylate glucose, fructose and mannose when supplemented with phosphoHPr. Three homogeneous II-A type proteins were isolated from the membranes: II-A^{Glc}, II-A^{Fru}, and II-A^{Man}. To reconstitute active Enzyme II, we needed another membrane protein, II-B, as well as a minor *E. coli* lipid, phosphatidylglycerol, and a bivalent cation. The II-B protein(s) is difficult to work with since it aggregates in the absence of detergent. Moreover, the only evidence for homogeneity is insufficient; it yields but a single band on electrophoresis (sodium dodecyl sulphate–polyacrylamide disc gel). If the protein is homogeneous, then it appears that a single II-B protein is shared by the three II-A proteins (Fig. 4). Also, the reformation of the active II-B complex requires the recombination of the II-B protein with the lipid and bivalent cation in a specific order; then, the complex becomes easily sedimentable. These results open up obvious possibilities for studying how this type of protein may be inserted into the bacterial membrane as well as for reconstituting the translocating system in artificial vesicles.

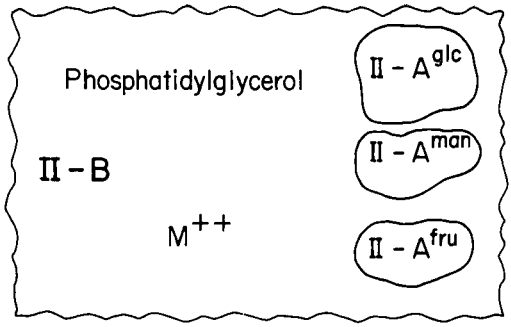

FIG. 4. The constitutive Enzyme II complex of membranes of *E. coli* or of *S. typhimurium*.

A second type of sugar-specific duplex, isolated from *S. aureus*, is responsible for the phosphorylation of lactose (at the C-6 position of the galactose). Factor III^{Lac}_{Sa} is a trimeric protein, containing three identical subunits of about 12 000 daltons (Hays *et al.* 1973). The subunits can each accept a phosphoryl group from phosphoHPr without requiring any additional protein, and in phospho-III^{Lac}, the phosphoryl group appears to be linked at the N-3 position of the imidazole ring in a histidyl residue. As noted before, the transfer of phosphoryl from phospho-III^{Lac}_{Sa} to lactose or its analogues requires the membrane protein, II-B^{Lac}_{Sa}, which we have not yet succeeded in isolating in soluble form. Nevertheless, studies on the binding of membrane preparations from a variety of mutants lead us to conclude that it is the II-B^{Lac} protein which binds and recognizes the sugar (Simoni & Roseman 1973).

A third type of sugar-specific duplex, III^{Glc}_{Ec}/II-B'_{Ec}, is described below.

GROUP TRANSLOCATION OF PTS SUGARS

Although Fig. 3 represents the catalytic function of the phosphotransferase system, it ignores an important component, the membrane. Suitable provision for that has been made in Fig. 5. The sugar is phosphorylated while it is being translocated across the cell membrane. This is the simplest model for the function of the phosphotransferase system in the group translocation of carbohydrates: it is consonant with all the available evidence variously obtained (for reviews see Roseman 1969, 1972). To my knowledge, no evidence tells against this simple model.

In the generalized model (Fig. 6), that is, if a sugar-specific protein duplex is called II, then different sugars ($S_1 \ldots S_n$) are translocated and concomitantly phosphorylated by the corresponding sugar-specific duplexes ($II_1 \ldots II_n$) using

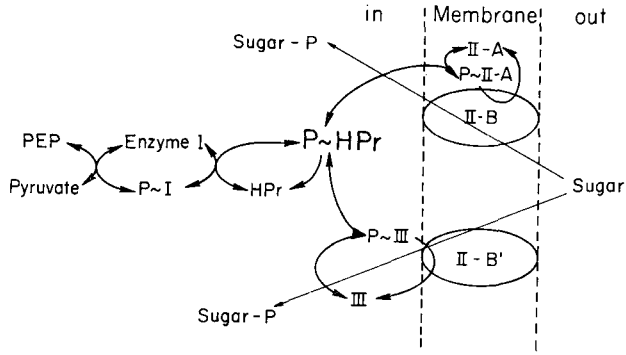

FIG. 5. Sugar translocation and concomitant phosphorylation by the phosphotransferase system.

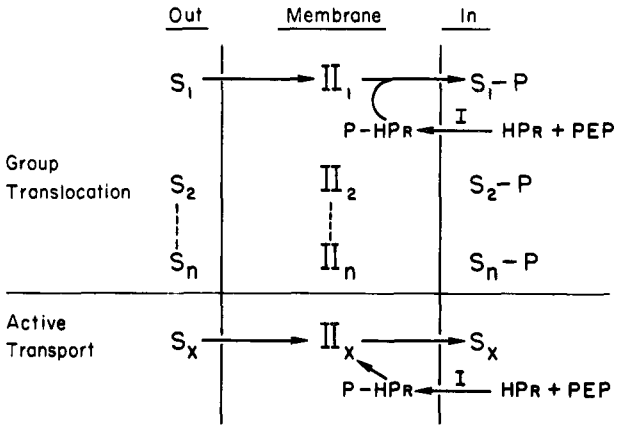

FIG. 6. General scheme for group translocation of sugars in wild-type cells by the phosphotransferase system: $S_1 \ldots S_n$ signifies different sugar substrates for the system; $II_1 \ldots II_n$ are the corresponding sugar-specific duplexes of the type illustrated in Figs. 4 and 5. There is no evidence that the phosphotransferase system participates in active transport (bottom of Fig.), but this scheme suggests a mechanism for such a process in accord with the model shown in Fig. 10.

energy and phosphoryl groups from phosphoHPr. This model may be tested by the use of mutants defective in one of the PTS proteins, since we can predict from the model that a defect in Enzyme I or HPr should affect the uptake of all PTS sugars, whereas a defect in one sugar-specific protein should affect the uptake of that sugar only. Using *S. aureus*, Simoni & I (1973) obtained precisely these results. I should emphasize that the intact system is necessary

for sugar uptake; for example, the Enzyme I mutants of *S. aureus* could not equilibrate internally with external sugar by simple or facilitated diffusion during the duration of our experiments.

THE TRANSPORT OF OTHER CARBOHYDRATES

I have so far concentrated on the enzymological properties of the phosphotransferase system and its role in the group translocation of sugars which are known to be substrates of the system. Although best known for its role in group translocation an increasing body of evidence suggests that the PTS also contributes to the regulation of the following physiological processes: the transport of some non-PTS sugars; the synthesis of the corresponding inducible catabolic enzyme systems and permeases; the control of the intracellular concentrations of cyclic AMP; and, possibly, the activities expressed by certain membrane-bound enzymes and chemotaxis. I shall now consider the regulation of transport of the non-PTS sugars.

A definition of PTS and non-PTS sugars is not possible without a specification of the organism. Present evidence indicates that all carbohydrates taken up by *S. aureus*, including lactose, are PTS sugars. However, in *E. coli* and *S. typhimurium*, some sugars are PTS sugars (glucose, fructose, mannose, hexitols, hexosamines and *N*-acetylhexosamines, β-glucosides, etc.) but others are not (lactose, maltose, melibiose, pentoses, glycerol, galactose, etc.). As Henderson & Kornberg describe (pp. 243–261), the latter group of compounds, as well as amino acids and other solutes, are presently thought to be transported by the process of co-transport, that is, in conjunction with a cation which moves down its electrochemical gradient with the solute by the relevant permease, thereby allowing the solute to accumulate against its concentration or the electrochemical gradient. Stock & I (1971) have reported that these organisms take up melibiose by a sodium co-transport system. The present view, formulated by Mitchell (reviewed by Harold 1972), is that essentially all these systems are proton co-transport systems. The evidence for cation co-transport of non-PTS solutes is substantial and implies that these permeases are separate and distinct from the phosphotransferase system having evolved independently of it in organisms such as *E. coli* and *S. typhimurium*. Is this conclusion correct?

In early studies, Enzyme I and HPr mutants were found to be unable to grow on or ferment a wide variety of non-PTS sugars, from which result it was incorrectly concluded that the phosphotransferase system functioned directly in the transport of these compounds. Later experiments, initiated by Gershanovitch *et al.* (1967) and followed by many others, showed that the problem was one of induction: these mutants were not normally inducible for the catabolic

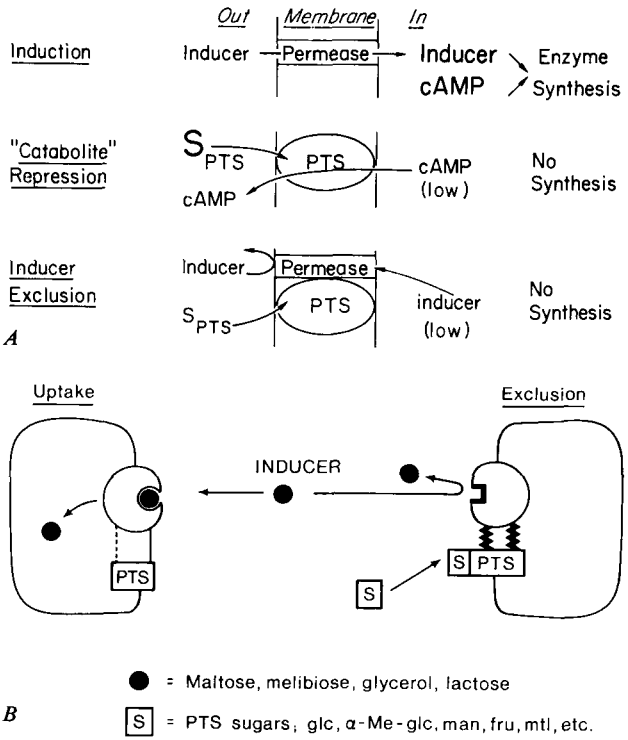

FIG. 7. Postulated mechanisms for the repression of synthesis of inducible enzymes by the phosphotransferase system: *(A)* catabolite repression and inducer exclusion; *(B)* inducer exclusion (*E. coli*, *S. typhimurium*).

enzyme systems and permeases required for utilization of the non-PTS sugars. The mutants are hypersensitive to repression and are particularly repressed by low concentrations of any PTS sugar added to the inducing medium. Two explanations have been offered to explain this type of PTS-mediated repression (Fig. 7). Pastan & Perlman (1969) suggested that this type of repression (Fig. 7*A*) resulted from a cellular efflux of cyclic AMP when a PTS sugar was added to the medium, which thereby prevented the synthesis of the inducible enzyme system. Saier's results (reviewed in Roseman 1972) suggest instead that the most important phenomenon is inducer exclusion. That is, the addition of very small amounts of PTS sugars to the inducing medium prevents the uptake of the non-PTS inducing sugar by the cell, thereby precluding induction (Fig. 7*B*).

The phenomenon was analysed with four non-PTS sugars (glycerol, melibiose, maltose and lactose) and many PTS sugars. For brevity, I shall describe the

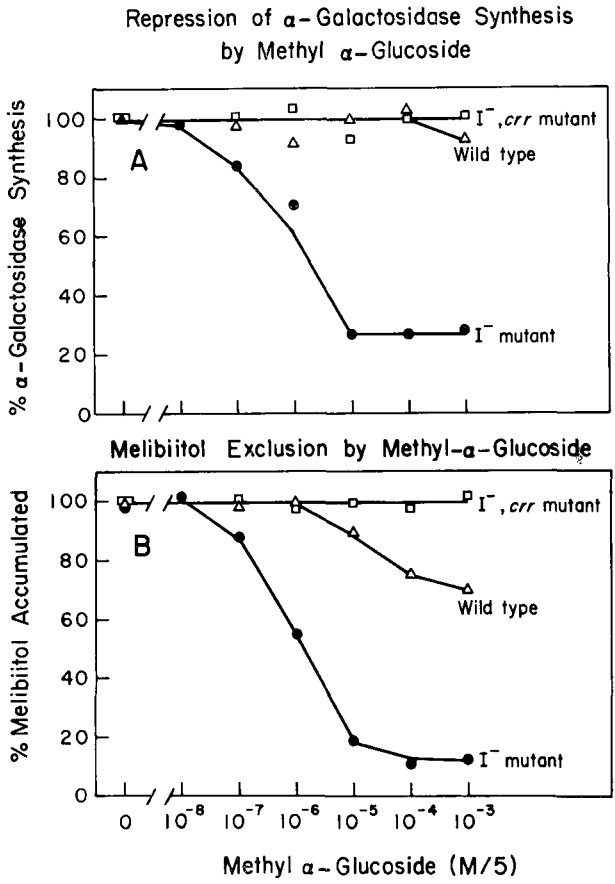

FIG. 8. Repression of synthesis of α-galactosidase and of inducer (melibiitol) uptake as a function of methyl α-glucoside concentration.

results with one PTS sugar, methyl α-glucoside (MeGlc). (*a*) The addition of 0.1–10μM-MeGlc immediately stops the uptake of the four non-PTS sugars (at 1 mmol/l) in Enzyme I and HPr mutants. Wild-type cells are also sensitive to the PTS sugars but much higher concentrations are required. (*b*) Uptake, not efflux, is inhibited. (*c*) Inhibition is observed equally in fully induced and in uninduced cells. (*d*) Methyl α-glucoside does not compete with the non-PTS sugars for the relevant permeases. (*e*) Uptake of the glucoside is not detectable in the Enzyme I mutants at concentrations where this PTS sugar exerts an immediate effect on the uptake of the non-PTS sugars. Furthermore, it is not metabolized. (*f*) Inhibition by a given PTS sugar requires a functional Enzyme II for that sugar.

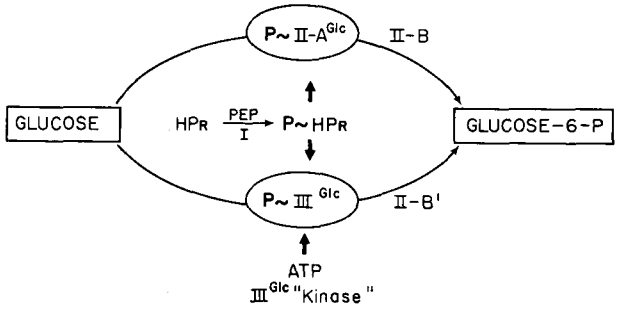

FIG. 9. Glucose phosphorylation. In *E. coli* and *S. typhimurium* two glucose-specific duplexes (Table 1) can phosphorylate glucose when supplemented with phosphoHPr.

These results, therefore, suggest that the phosphotransferase system somehow regulates the uptake of non-PTS solutes, and the phenomenon is most strikingly exhibited by Enzyme I and HPr mutants. Additional evidence for the regulatory role of the system in the uptake of non-PTS solutes is obtained by genetic methods. Repression mediated by the PTS and the hypersensitivity of Enzyme I mutants to PTS sugars can be overcome by the introduction of an additional point mutation into these cells, the mutation being designated *crr*. Enzyme I *crr* double mutants totally resist the effects of PTS sugars, for example, 0.02M-MeGlc (Fig. 8). The nature of the *crr* mutation is, therefore, of prime importance in any attempt to determine the mechanism of PTS-mediated repression and inducer exclusion.

The *crr* gene has been mapped (Cordaro & Roseman 1972) and lies just outside of the *pts* operon, which contains the structural genes for HPr and Enzyme I. The more important question concerns the biochemical defect that results from the *crr* mutation. We have so far found only one change in cells containing this mutation. These cells do not contain the PTS protein, III^{Glc}. In *E. coli* and *S. typhimurium*, two phosphotransferase systems can take up glucose (or methyl α-glucoside). One, the $II\text{-}A^{Glc}$/II-B system, I have described above. The second involves III^{Glc} and a separate membrane protein, II-B′. In the enzymic assay, the two systems function as shown in Fig. 9, and glucose or MeGlc can be phosphorylated if either duplex is provided with phosphoHPr.

Dr W. Kundig has purified III^{Glc} to apparent homogeneity and has extensively studied this protein. It exhibits an apparent molecular weight (by gel filtration) of about 20 000 daltons, and contains 3–4 subunits of 5000–7000 daltons. The purified preparation shows an extremely potent activity of hexose 6-phosphatase, which is inhibited by fluoride ions, and is specific for hexoses of the D-*gluco* or D-*manno* configurations. As far as we can tell, the properties of this phosphatase

differ from any previously isolated from bacterial cells. Current experiments are aimed at determining whether the phosphatase is intrinsically associated with the same protein that shows III^{Glc} activity. When the intact protein is heated for brief periods at 70 °C, it completely loses all phosphatase activity but retains full transferase activity, and this process is simultaneously accompanied by dissociation to its subunits.

The protein III^{Glc} accepts the phosphoryl group from phosphoHPr, but by contrast with all other phosphoproteins of the PTS so far characterized, which are phosphohistidylproteins, in phosphoIII^{Glc} the phosphoryl group is linked to a carboxy group, in exact analogy to the Na^+,K^+-activated animal ATPase described by Glynn & Karlish (pp. 205-220).

A critical question must now be examined by genetic techniques, and concerns the *crr* mutation. Wild-type cells, and particularly Enzyme I and HPr mutants, are unable to take up four non-PTS sugars at normal rates when such cells are simultaneously exposed to very low concentrations of any PTS sugar. Introduction of a point mutation, *crr*, makes such cells totally insensitive to the PTS sugars. The role of the *crr* gene is, therefore, of paramount importance. If it is a regulatory gene which controls the synthesis of more than one protein, amongst them III^{Glc}, then no clear interpretation of the present results can now be offered. If, on the other hand, *crr* is the structural gene for III^{Glc}, then this protein somehow regulates the uptake of the non-PTS sugars. Should the latter prove to be true, then we must ascertain how III^{Glc} and other PTS proteins execute this regulatory role. Again, no precise explanation can now be offered, but one series of preliminary experimental results offer a clue.

The entire discussion just given has been restricted to the phosphotransferase system, which serves to transfer phosphate from phosphoenolpyruvate to sugar. Are there any variants of this system? We now know of several such variants, perhaps the most interesting and potentially important is that shown in Fig. 9. Preliminary results indicate that phosphorylation of III^{Glc} can be catalysed by proteins other than Enzyme I/HPr (called 'kinase' in Fig. 9) and, much more importantly, that the phosphoryl donor is ATP. Should these results be confirmed, they suggest alternative pathways for the transfer of phosphoryl groups with some of the PTS proteins (but not Enzyme I and HPr), and it should not be difficult to speculate how these alternative pathways are either directly involved or at least regulate the transport of non-PTS solutes. The earliest models for facilitated diffusion and active transport (Fig. 10) suggested that active transport resulted from a cycle of phosphorylation and dephosphorylation by the membrane-bound protein carrier. The phosphoprotein had a low affinity for the solute, but the dephosphorylated protein had a high affinity for the solute. This simple model has been confirmed in the

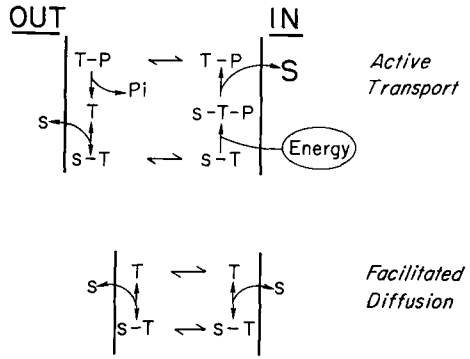

FIG. 10. Early model of facilitated diffusion and active transport. The solute-specific transport protein is designated *T*, the solute *S*. In active transport, the protein of the protein–solute complex is phosphorylated. The phosphoprotein has a low affinity for the solute and the complex dissociates. The cyclic phosphorylation–dephosphorylation of the protein acts as a solute pump.

mechanism by which the membrane-bound ATPase pumps sodium ions, although the precise details of mechanism are more complex. The phosphotransferase system is, after all, also a system using phosphoproteins, and it should not be too difficult to imagine that the process of evolution has resulted in modification of the system to meet new demands, rather than in the development of entirely new transport systems. I suggest that the general mechanism for active transport of essentially all solutes will prove to be conceptually similar to that found for the Na^+,K^+-ATPase and for the phosphotransferase system, that is it will involve phosphoproteins.

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Discussion

E. Krebs: Does the phosphotransferase system exist in mammalian cells? Historically, the idea that sugars needed to be phosphorylated before they could be taken up by cells was a common hypothesis.

Roseman: Weiser & Isselbacher (1970) reported the existence of this system in intestinal cells, but that has to be confirmed. Because we are dealing with phosphoenolpyruvate it is difficult to demonstrate this system in crude extracts. For example, we spent three months trying to find the system in extracts in *Salmonella typhimurium* and as long in *Staphylococcus aureus*, despite the fact that the system is extremely active in both organisms. We have done experiments with animal cells but we thought that we were observing transfer of the phosphoryl group by the widely distributed glucose-6-phosphatase. We ran into many artifacts and were never convinced that the system was present.

E. Krebs: Is the translocation of cyclic AMP affected by this system?

Roseman: Yes. Discussion of the topic of catabolite repression would take too long to detail but, in summary, when Makman & Sutherland (1965) added glucose to bacterial cells, cyclic AMP immediately flowed out of the cell. Pastan & Perlman (1970), who had investigated the repression mediated by the phosphotransferase system, suggested that this was the mechanism of repression. However, they did not measure the concentration of cyclic AMP. Although in some cells it may be related to the concentration of cyclic AMP, we believe that the inducer–exclusion phenomenon is the more important part of the repression mediated by the phosphotransferase system at least in *S. typhimurium*.

Whatley: Why doesn't the phosphotransferase system sugar added to prevent induction itself get taken up?

Roseman: We cannot detect any uptake of, say, methyl α-glucoside in Enzyme I mutants unless we use extremely high concentrations. To get measurable translocation of methyl α-glucoside (or any phosphotransferase system sugar) we need the intact system. The leaky Enzyme I mutants can pick up the glucoside at sufficiently high concentrations; the K_m is about 10^{-3} mol/l for the wild-type cell. However, the exclusion effect is observed at 10^{-7} mol/l in these Enzyme I mutants, where we simply cannot see any uptake at all.

Whatley: If you used glucose instead of methyl glucoside, would you get this effect?

Roseman: We do: uptake is inhibited. The advantage of methyl glucoside is that it is not metabolized further than being phosphorylated by the phosphotransferase system.

Lipmann: I was fascinated by your observation of phosphoenolpyruvate as a donor, for it seems to be the only well documented donor known in addition to ATP. You glossed over this first enzyme, but that is a key enzyme because it triggers the whole chain of reactions.

Roseman: Using the 'homogeneous' enzyme, we have demonstrated direct phosphorylation: transfer of phosphate from phosphoenolpyruvate to Enzyme I; then with the phosphoEnzyme I, transfer to HPr. We don't know the size of the subunits of Enzyme I, or even whether they are identical. It is extremely sensitive to inhibitors specific for thiols. However, we simply do not understand many functions of Enzyme I. I have referred to one of them. Mutants defective in the protein are not only defective in taking up and using PTS sugars, but suffer from an inability to be normally inducible for enzymes required for the use of certain non-PTS sugars. Thus, I should not be at all surprised to find that some of the subunits of the protein regulate its catalytic action and, further, that the protein or phosphoprotein itself regulates other processes in the cell.

Lipmann: Do both the II^{Glc} and III^{Glc} enzymes carry phosphate groups, one on the carboxy group and the other on histidine? Does the charging of the II enzyme specifically depend on a phosphoenolpyruvate pathway?

Roseman: The point I tried to make is illustrated in Fig. 1. Glucose can be phosphorylated by two separate phosphotransferase systems; in both cases the phosphoryl group is derived from phosphoHPr. One system is the II-A/II-B

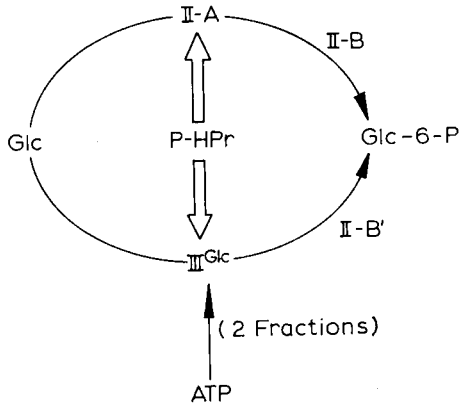

FIG. 1 (Roseman). Two phosphotransferase systems for the phosphorylation of glucose.

membrane duplex. The other involves the soluble protein, III^{Glc} and a separate membrane protein, II-B′. As far as we know, the II-A/II-B system is entirely linked to phosphoenolpyruvate. The III^{Glc}/II-B′ duplex, on the other hand, is capable of using two phosphate donors, phosphoHPr and ATP. Our (preliminary) findings with the latter system indicate a branch point in the phosphotransferase system not involving Enzyme I and HPr but at least two other protein fractions. We are particularly interested in this type of modification of the system since it has always appeared possible that its very complexity should allow modification of the PTS.

We are currently studying yet another variant of the system involving a protein which serves in place of HPr but is neither HPr nor a mutant form of HPr itself.

Lipmann: Can this phosphotransferase revert phosphoryl to pyruvate from the phosphate enzyme?

Roseman: Yes. The equilibrium constant has been determined in both directions.

Hess: Is hexokinase functioning in the transport mechanism and could there be a hexokinase bound to the membrane?

Roseman: Two glucose kinases are found in these kinds of cells: a glucokinase and mannofructokinase which also acts on glucose but not so well. The situation appears to be complex. Some time ago, hexokinase was proposed as a factor in sugar transport (as Dr E. Krebs mentioned). We can now prepare mutants lacking the kinase and which still contain an intact phosphotransferase system. The 'missing bacterial kinases' have now been identified as the phosphotransferase system.

Whatley: How does all this apply to chemotaxis?

Roseman: Adler & Epstein (1974) have recently demonstrated that the PTS is involved in chemotaxis for PTS sugars. Mutants defective in a sugar-specific protein do not exhibit chemotaxis for that sugar.

Whatley: Does this imply that the organism has a distinguishable front and rear end?

Roseman: There does appear to be polarity or asymmetry in bacterial cells in migration toward an attractant.

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The active transport of carbohydrates by *Escherichia coli*

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Abstract The active transport of carbohydrates by *Escherichia coli* is discussed with particular reference to (1) identification of an uptake process as 'active transport', (2) nature and control of transport proteins, and (3) mechanisms of energy transduction.

(1) The use of substrate analogues, of mutants blocked in metabolism and of subcellular vesicles in the isolation of the transport process from interference by subsequent metabolic reactions is described. Criteria are outlined for establishing that the solute is taken up against a concentration gradient and that this is energy-dependent. Three types of poisons for energy systems that act primarily on respiration, on ATP formation and as uncoupling ('proton conducting') agents are considered.

(2) Methods are described for the selection of mutants impaired in the active uptake of specific carbohydrates.

(3) Results show that the uptake of galactose, D-fucose and arabinose by appropriate strains of *E. coli* is inducible, specific and accompanied by proton uptake. Such and other data support a model based on a chemiosmotic theory of active transport.

Whereas mammalian cells are constantly bathed in a medium of relatively invariant composition, bacteria have no such assured milieu. It is thus appropriate, and a source of teleological satisfaction, that part of the energy derived from the microbial catabolism of carbohydrates is diverted to facilitate the uptake of carbohydrates into the cells. This is achieved by two main mechanisms. The sugar may be chemically modified on uptake, as is the case with the phosphoenolpyruvate-dependent phosphorylation of hexoses (see Roseman, this volume); however, this process appears to be largely confined to facultative anaerobic, or 'micro-aerophilic', bacteria (Romano *et al.* 1970) and is thus not of general utility. A more prevalent process is that described as 'active transport', in which the material taken up appears chemically unmodified

inside the cell and (if its catabolism is prevented) may do so at a much higher concentration than that present outside.

Our main aim in this paper is to discuss the problems in and experimental methods for elucidating the molecular mechanism of active transport, using the uptake of carbohydrates by *Escherichia coli* as example. We shall pose three basic questions:

(A) How is the translocation of a molecule across the cell membrane of an organism identified as 'active transport'?

(B) How are proteins involved in active transport recognized?

(C) How is energy transduced into the active transport process?

It is appropriate in the context of this symposium to discuss the transduction of biological energy into vectorial processes, since these questions bear directly on the general mechanisms of biological energy conservation.

We shall assume that there is a 'high energy intermediate' common to membrane-located processes that generate and use biological energy. This high energy intermediate may be a particular chemical compound (Slater 1953), a conformational state of a protein (Boyer 1967) or an electrochemical gradient of ions (Mitchell 1966). More and more evidence is accumulating to support the last possibility, and we shall describe this evidence in some detail. However, lack of evidence for alternative explanations by no means eliminates the operation of conformational states and chemical intermediates. It seems inherently plausible that a protein conformational change is involved in the process of active transport: the evidence discussed later (p. 250) shows that proteins mediate this process, and they must do so in a way that facilitates vectorial translocation of substrate (see also Boyer & Klein 1972; Wilson *et al.* 1972). For the purposes of the present discussion, Fig. 1 postulates, as predicted by the chemiosmotic theory of Mitchell (1963), that the high energy intermediate is generated simultaneously with, or is identical to, translocation outwards of protons (or the experimentally indistinguishable translocation inwards of hydroxy ions). Those to whom this 'chemiosmotic' viewpoint is untenable may replace the proton gradients in the diagram with $\sim$ (Slater 1953).

The components illustrated in Fig. 1 are the respiratory chain, ATP synthase, and the nutrient uptake systems—all of which are membrane bound (Harold 1972). In aerobic conditions the respiratory chain oxidizes substrates and presumably generates the high energy intermediate; in *E. coli* protons are transported outwards during this process (West & Mitchell 1972; Lawford & Haddock 1973) and an electrical potential, positive outside, is established (Lombardi *et al.* 1973; Hirata *et al.* 1973). The energy required for the accumulation of sugars or for the synthesis of ATP is derived from this high energy

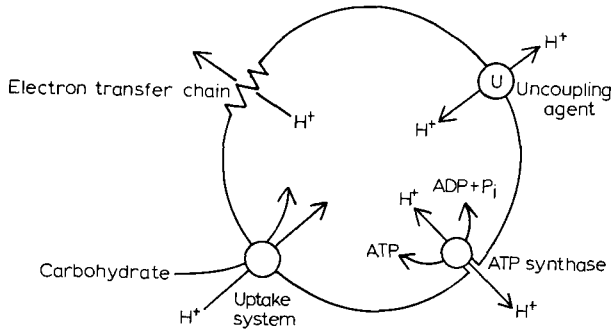

FIG. 1. Scheme for chemiosmotic coupling of energy-linked processes. The large circle represents the cytoplasmic membrane of a single bacterial cell—the cell wall has been omitted for clarity.

state (Mitchell 1966, 1970, 1973). Good evidence exists that many energy-requiring processes of bacteria, such as active sugar transport and the synthesis of ATP, are associated with the inward transport of protons and collapse of the electrical potential (for review, see Harold 1972; Mitchell 1973; see also Rosen 1973; West & Mitchell 1974). Fig. 1 also shows how proton-conducting ('uncoupling') agents can short-circuit the maintenance of this electrochemical gradient for transport or ATP synthesis.

CHARACTERIZATION OF ACTIVE TRANSPORT

Active transport may be distinguished from group translocation or facilitated diffusion by three properties: the substrate appears inside the organism in a chemically unchanged form; the substrate is accumulated against a concentration gradient; and metabolic energy is expended to accomplish the accumulation. Several experimental tests can be applied to distinguish these properties, and these are outlined below.

Isolation of a transport reaction

The uptake of a food material by microorganisms is usually measured with substrates labelled with a radioactive isotope, normally through determinations of the time course of appearance of the label in the cells, separated from their ambient medium. If the substrate thus used is metabolically usable by the organism, its initial uptake is rapidly followed by its catabolism and by use of the catabolic fragments for cellular syntheses. Clearly, an unambiguous study

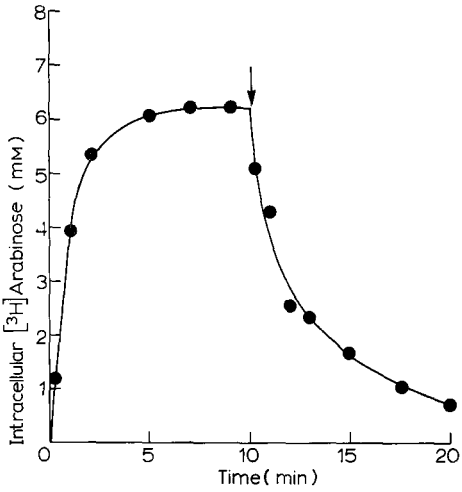

FIG. 2. The energized uptake of [^{3}H]arabinose into *E.coli* SB 5313, and the effect of subsequent addition of unlabelled arabinose. Bacteria (2.2 mg dry mass) were incubated aerobically for two minutes at 25 °C in 150mM-KCl, 2mM-glycylglycine buffer, pH 6.8 (1.5 ml volume). Arabinose (75 nmol, specific activity 0.2 μCi/μmol) was added, and samples were taken and analysed for radioactivity as described by Morgan & Kornberg (1969). Unlabelled arabinose (1.8 μmol) was added at the point indicated by the arrow.

of the uptake process requires that means be found for isolating that uptake step from subsequent cellular processes. There are three techniques available for doing so.

(i) *Use of substrate analogues.* The carbohydrate transport systems of *E. coli* often transport chemical analogues as well as the normal substrate and in many cases the analogues are not further metabolized. The classic example is methyl β-D-thiogalactoside, a substrate for the system that transports β-galactosides but not for the enzyme (β-galactosidase) that catalyses their hydrolysis (Rickenberg *et al.* 1956). Similarly, methyl β-D-galactoside and D-fucose have been used extensively in the isolation of the activity of galactose transport systems (Ganesan & Rotman 1966); D-fucose is also transported by the arabinose transport system(s) (Novotny & Englesberg 1966).

(ii) *Use of mutants.* If a mutant can be obtained which is unaffected in the active transport of a particular substance but is impaired in the first enzyme whereby that substance enters metabolic pathways, then the uptake system can be studied in isolation from possibly interfering subsequent events. An example of the use of such a mutant is given in Fig. 2. In this, *E. coli* strain SB 5313,

which differs from its wild-type parent in lacking arabinose isomerase (the first enzyme of arabinose catabolism: Hogg & Englesberg 1969) is shown to take up isotopic material rapidly when a washed suspension of the cells, induced with arabinose, is incubated with [^{3}H]arabinose. That the material thus taken up is not incorporated into cell components but is accumulated as such is shown by its rapid displacement by unlabelled arabinose. Cells not induced by growth in the presence of arabinose do not exhibit this behaviour.

However, although the availability of mutants of this type provides unambiguous information on the uptake process, information can also be reliably obtained with wild-type cells provided that the time intervals chosen for sampling are brief enough. Horecker *et al.* (1960) observed that the initial rate at which labelled galactose was taken up by wild-type *E. coli* was identical to that at which galactose was taken up by a mutant devoid of galactokinase activity but, whereas galactose accumulated in the mutant, no such accumulation was detected in the parent organism. Similarly, mutants of *E. coli* (Pouysségur *et al.* 1974) and of a thermophilic bacillus (S. S. Bungard & H. L. Kornberg, unpublished results) devoid of gluconate kinase take up labelled gluconate at initial rates identical to those observed with their wild-type parents. It is thus likely that the uptake process imposes the rate-limiting step in the use of these substrates.

(iii) *Use of subcellular vesicles.* Membrane vesicles of *E. coli* prepared by the method of Kaback (1971) retain the ability to take up and accumulate a number of compounds at rates and to the extent seen with intact cells. In the preparation process the enzymes of further metabolism are eliminated. However, it must also be remembered that 'binding proteins', which are known to participate in some chemotactic and transport reactions (Adler *et al.* 1973; Boos 1972), are also lost.

Vesicles have been particularly useful in cases where the metabolic steps following entry have not been sufficiently well characterized to identify mutants, or where such mutants have been difficult to isolate. Since such vesicles operate in the absence of cell walls and cytoplasmic enzymes, they are particularly suitable for investigation of the role of energy donors, inhibitors and other agents that might not otherwise penetrate into the intact bacteria or be rapidly destroyed in them; their use has even allowed the activity of a mutant transport system to be restored by the addition of an enzymic component purified from wild-type cells (Kaback 1974).

Identification of the internal product of the transport step

Once further metabolism has been eliminated, radioisotope-labelled substrate may be added outside the cells, which are then separated from the medium by centrifugation or filtration so that the amount accumulated inside may be determined. The internal labelled compound(s) can be extracted and identified chemically by appropriate procedures. If a single compound identical to that applied outside the cells is found inside, then the process is unlikely to be group translocation but may be active transport *if* the internal concentration of the accumulated material is significantly higher than the concentration of the material outside the cells.

Calculation of the internal concentration of substrate taken up

It is obviously essential that any labelled substrate adhering to the cells, and any that has diffused into the periplasmic space but has not crossed the membrane, be removed before attempts are made to calculate internal concentrations. The usual procedure for doing so is to filter or centrifuge the cells rapidly and to wash them with an appropriate fluid devoid of the labelled substrate.

It is necessary to determine that the method of harvesting the cells does not cause or allow leakage of the accumulated material from the cells, and that neither the composition nor the temperature of the washing fluid causes or allows the loss of accumulated materials. (For example, bacteria that retain accumulated β-galactosides when washed with salt solutions at 30 °C may lose most of this material if washed at 0 °C.)

Calculation of the internal concentration of an accumulated material also necessitates knowledge of the specific activity of the material present externally and the amount of cellular water. Measurements with *E. coli* (Roberts *et al.* 1955; Winkler & Wilson 1966) indicate that the dry mass of the organism is about 25% of its wet weight, and that 1 mg dry mass may be taken to be equivalent to 3 μl of cell water.

Energy dependence

By definition, active transport and group translocations depend on the input of metabolic energy. Thus, interference with this energy provision should also impair these processes. However, as mentioned previously, the phosphoenolpyruvate-dependent phosphotransferase system has been found generally in

organisms that cleave carbohydrates by routes leading to the (anaerobic) production of phosphoenolpyruvate, whereas active transport is associated primarily with oxidative metabolism. Hence, use of energy poisons can reveal not only that the uptake of a particular substrate is energy-dependent, but the selective effects of different poisons can provide additional means of distinguishing active transport from phosphoenolpyruvate-dependent group translocation.

Three types of poisons have been particularly useful in providing information on the mechanism of energy coupling to active transport:—

(i) *Inhibitors of respiration.* Hydroxyquinoline *N*-oxide, cyanide and lack of oxygen all prevent the generation of the high-energy intermediate from electron transfer reactions (Harold 1970, 1972; Kaback 1974); in *E. coli*, they all inhibit active transport of sugars, but usually do not abolish it (Klein & Boyer 1972). The reason for incomplete inhibition appears to be that ATP generated by glycolysis acts as an alternative energy source.

(ii) *Inhibitors of ATP formation.* Dicyclohexylcarbodiimide (DCCD), the antibiotic Dio-9 and inorganic arsenate, all prevent formation of ATP by influencing the ATP synthase directly (Harold 1970, 1972; Klein & Boyer 1972). Again, these compounds rarely inhibit energized transport completely but, when applied in combination with inhibitors of respiration, active transport can be abolished.

These observations provide good evidence that either respiration or ATP hydrolysis can independently provide energy for active transport. Further support derives from the finding that mutants lacking a functional ATPase can carry out active transport in aerobic, but not in anaerobic conditions (Schairer & Haddock 1972; Yamamoto *et al.* 1973; Rosen 1973), and that in an obligate anaerobe, *Streptococcus faecalis*, DCCD or Dio-9 prevent active transport of β-galactosides (Harold *et al.* 1969*a*,*b*).

(iii) *Uncoupling agents.* Classically, uncoupling agents prevent the utilization of the high energy intermediate, generated from respiration, for ATP synthesis. Consequently, such agents should also, and do, prevent input of the intermediate into active transport of sugars by *E. coli* (for review see Harold 1972). 20–30 mM-Sodium azide or 2mM-2,4-dinitrophenol have been used widely, but much lower concentrations (20 μmol/l) of uncouplers such as tetrachlorosalicylanilide or carbonyl cyanide *m*-chlorophenylhydrazone are equally effective. In anaerobic conditions they also prevent the high energy intermediate generated by ATP *hydrolysis* from supporting sugar transport, even though high con-

centrations of ATP may be maintained in the cell (Pavlasova & Harold 1969; Harold 1972).

NATURE AND CONTROL OF TRANSPORT PROTEINS

The procedures discussed so far provide experimental techniques for the definition of the kinetic and energetic parameters of active transport. Such kinetic studies reveal that the basic features of these processes are akin to those of enzyme-catalysed reactions: high specificity for substrate and only relatively slight latitude in the chemical structure of a characteristic linkage or group if that substrate is to be recognized and translocated. Similarly, many non-metabolizable analogues of substrates are taken up even better than the 'normal' substrate; others, suitably modified to enhance their binding to a transport protein, can act as highly selective and powerful inhibitors of substrate uptake. Indeed, the analogy with enzymic reactions extends further. The kinetics of accumulation of appropriate analogues by wild-type cells, or of substrates by mutants blocked in their catabolism, can be described in a manner identical in form to the Michaelis–Menten relation (Rickenberg *et al.* 1956; Kepes 1960; Winkler & Wilson 1966). Moreover, if both of two analogues have affinity for a binding site for transport, the addition of one to cells already equilibrated with the other will cause the rapid and virtually complete displacement of that other (Rickenberg *et al.* 1956).

If the procedures described above are applied to cells grown in a variety of different conditions, they will provide information also on a second type of specificity, analogous to that observed with inducible enzymes. Particular active transport systems are usually present in significant amounts only when the cells have been previously exposed to substances containing the appropriate chemical groups. Moreover, as was shown many years ago (Kogut & Podoski 1953; Barrett *et al.* 1955; Cohen & Monod 1957), the differential synthesis of uptake systems, like that of inducible enzymes, is abolished or arrested by ultraviolet irradiation, by amino acid analogues and by other agents known to interfere with protein synthesis. Polar mutants, which result in the premature termination of polypeptide chains, have been described for the uptake system for β-galactosides that is specified by the *lac y* gene (Zipser 1970): together with the other evidence, this leaves little doubt that the components that effect the passage of lipid-insoluble carbohydrates across the membranes are proteins. Bacteria must carry in their genomes base sequences that specify the structure of these transport proteins and base sequences that determine when this structural information is expressed.

But the procedures described before cannot give any direct information on

the identity of specific transport proteins. Ideally, such information might be obtained from study of the isolated proteins. However, only in one instance—the β-galactoside transport system—has an unique membrane protein ('M protein') been isolated and identified as the product of the *y* gene that specifies the permeation step (Fox & Kennedy 1965; Fox *et al.* 1967), and only in this instance have differences been reported to occur during the uptake of β-galactosides in the accessibility of an essential –SH group (Yariv *et al.* 1969), differences which accord with postulated conformational changes expected of a carrier protein. In the absence of other available techniques, information on the nature and regulation of proteins involved in active carbohydrate transport must be sought either through kinetic analysis of cells and vesicles or by perturbing the normal system—i.e., by studying bacterial mutants that are altered in these properties. Here, we must take care to distinguish between mutants that lack a function of a membrane protein and mutants that are altered in 'binding proteins'. Some binding proteins have been isolated from Gram-negative bacteria by subjecting the organisms to cold osmotic shock (Heppel 1969) but the available evidence indicates that they are periplasmic (i.e., located outside the cytoplasmic membrane; Heppel *et al.* 1972) and their role in the active transport of such carbohydrates as they can bind is not clear. Whatever that role is, it is not likely to be essential to the translocation process.

Recognition of transport proteins

Mutants impaired in membrane proteins involved in the uptake of particular carbohydrates can be obtained after chemical mutagenesis or by selection of organisms resistant to some otherwise toxic substrate or substrate analogue. This latter technique is particularly useful for the selection of mutants specifically impaired in uptake processes: it relies on the simple principle, often enunciated by the cautious traveller, that the best way to avoid food poisoning is not to eat. Conditions have to obtain in which either a normal food material A, or an analogue A′ taken up by the same uptake system, inhibits the utilization of some other nutrient, B; mutants that can now tolerate the inhibitory agents A or A′ during growth on substance B often do not now take up A and A′, or occasionally, over-produce a transport system for substance B. Thus, for example, pyruvate and phosphopyruvate are strong inhibitors of isocitrate lyase (EC 4.1.3.1), the first enzyme of the glyoxylate cycle (Kornberg 1966); the growth on acetate of *E. coli* mutants impaired in anaplerotic enzymes (and that, therefore, lack the ability to channel these C_3-compounds into the tricarboxylic acid cycle) is consequently arrested by the provision of these materials or of components catabolized to them. We made use of this phen-

omenon to isolate mutants specifically impaired in the uptake of pyruvate (Kornberg & Smith 1967), of hexoses and of hexose-phosphates (Kornberg & Smith 1969); we also obtained mutants impaired in the uptake of gluconate. In an analogous manner, mutants impaired in the uptake of C_4-dicarboxylic acids have been selected as colonies whose growth on acetate is resistant to fluoromalate or D-tartrate (Kay & Kornberg 1971).

In the second type of process, we took advantage of the well known phenomenon that the accumulation of phosphorylated sugar causes growth stasis but is apparently not lethal (for references, see Ferenci & Kornberg 1973). In the simplest application of this, we used an analogue of some food material that is a substrate for the transport process but not for metabolism. If the uptake of such an analogue leads to the formation and accumulation of a phosphorylated derivative, the toxic effect is observed. This method is particularly useful in obtaining mutants of the phosphoenolpyruvate-phosphotransferase system (Kornberg 1972), since in that system the analogue is necessarily taken up and accumulated in a phosphorylated form. However, a variant of this technique is useful also for the isolation of mutants impaired in active transport. Here, it is essential to use organisms that are already impaired in some catabolic step and that consequently accumulate one or more phosphorylated intermediates if exposed to the appropriate substrate. For example, *E. coli* uses gluconate predominantly through the Entner–Doudoroff pathway. Mutants blocked in component steps of that pathway, such as in the aldolase that catalyses the cleavage of 3-deoxy-2-oxo-6-phosphogluconate (KDPG) to pyruvate and glyceraldehyde 3-phosphate, accumulate KDPG in the presence of gluconate and no longer grow on a variety of otherwise usable nutrients if gluconate is also present (Faik *et al.* 1971). Mutants can be selected that now tolerate gluconate because they no longer take it up and hence no longer convert it into the still toxic KDPG (Faik & Kornberg 1973). Similarly, the growth on glycerol of mutants devoid of glucosephosphate isomerase (*pgi*: EC 5.3.1.9) and glucose-6-phosphate dehydrogenase (*zwf*: EC 1.1.1.49) is arrested when glucose or glucose 6-phosphate are added to the medium, since the latter cannot be catabolized and consequently accumulates. Further mutants can now be obtained whose growth is no longer inhibited by glucose 6-phosphate but is still sensitive to glucose: such mutants are unimpaired in their ability to take up glucose but no longer catalyse the active transport of glucose 6-phosphate and of other hexose phosphates.

Isolation of regulatory mutants

Mutants affected in the regulation of synthesis of proteins involved in active

transport have been obtained in a variety of ways. Isolation of organisms with genetic dysfunctions that extend beyond known structural elements can yield mutants that constitutively synthesize enzymes of, for example, catabolism of lactose (Jacob *et al.* 1964) and of gluconate (Zwaig *et al.* 1973), which include the uptake systems for lactose and for gluconate; the existence of such mutants points to the close association of structural and regulatory genes in at least these two cases. Mutants similar to the latter type have been obtained in attempts to isolate temperature-sensitive revertants of mutants impaired in gluconate utilization to growth on gluconate (B. Bächi & H. L. Kornberg, unpublished results).

Advantage has also been taken of the fact that some substrates of an active transport system are not inducers of that system. For example, fructose 1-phosphate is taken up by the uptake system for hexose phosphates but does not induce its synthesis: *E. coli* mutants selected for their ability to grow rapidly on fructose 1-phosphate as sole carbon source are thus likely to form this active transport system constitutively (Ferenci *et al.* 1971).

Fine control of active transport

Evidence is accumulating that the proteins involved in the active transport of carbohydrates possess not only a 'catalytic' site, which reacts specifically with the carbohydrate to be taken up, but also 'regulatory' sites that can interact with other carbohydrates that are not substrates for transport by that protein. Holmes *et al.* (1961) noted that glucose inhibited the hydrolysis of *o*-nitrophenylgalactoside (ONPG) by intact *E. coli* strain B but not its hydrolysis by cells rendered permeable by treatment with toluene. Similarly, the addition of glucose and of some non-catabolizable glucose analogues strongly inhibited β-galactoside uptake by glucose-grown suspensions of *E. coli* that were i^- (and hence constitutive for β-galactoside uptake) (Kepes 1960; Winkler & Wilson 1967). This use of glucose preferentially to other carbohydrates is not confined to lactose but has been observed with sugars taken up by active transport (such as maltose, xylose, arabinose and galactose; McGinnis & Paigen 1969) as well as with sugars taken up by group translocation (Kornberg 1972, 1973); the phenomenon has been given the general term 'catabolite inhibition' by McGinnis & Paigen (1969). Studies with a variety of mutants (McGinnis & Paigen 1969, 1973; Paigen & Williams 1970; Kornberg 1972, 1973) showed that this inhibition takes place before the first catabolic product is formed from glucose: the inhibitory effect is associated either with the intracellular concentration of glucose 6-phosphate (as appears to be the case with the 'fine control' of the phosphoenolpyruvate-phosphotransferase-mediated uptake of

fructose: Kornberg 1972, 1973) or, possibly, with the diversion of energy for the 'active transport' process into the phosphotransferase reaction. Although the mechanism is not yet clear, it is again a source of teleological satisfaction that enteric bacteria, which in their normal habitat are likely to encounter a number of sugars simultaneously, can benefit from this 'fine control' over the activity of carbohydrate uptake systems in order to regulate both the sequence in which sugars are utilized and the rate at which the chosen sugar is taken into the cell.

MECHANISMS OF ENERGY TRANSDUCTION

The model described by Fig. 1 was largely derived from the proposals of Mitchell (1970, 1973) and is consistent with the effects of the three types of inhibitor discussed before (pp. 249–250). It implies that the postulated high energy intermediate (whatever its nature) can be generated not only by respiration but also by hydrolysis of ATP, and can in turn drive not only active transport but also synthesis of ATP. This further implies that the sources of energy for the generation of the high energy intermediate, and the work done through its use, are reversibly linked.

It is also implicit in this model that the efficiency with which electron flow is coupled to active transport will depend on the rate of generation of high energy intermediate and on the inherent efficiency of a particular transport system to use it. Thus, we should expect that the relative efficiencies with which one electron donor energizes the active uptake of different substrates are the same as those with which another electron donor promotes the uptake of the same substrates. Furthermore, if two different electron donors produce similar rates of oxidation and a similar extent of reduction of the electron transfer components, they should energize a single transport system with similar efficiency. However, the extensive studies by Kaback and his colleagues on transport processes in subcellular vesicles show that different electron donors promote to widely different degrees the uptake of any one substrate and that a particular electron donor promotes to different degrees the uptake of different substrates (for review, see Kaback 1971, 1974). The chemiosmotic mechanism portrayed in Fig. 1 does not adequately account for these observations. Since D-lactate is the physiological electron donor of maximum efficiency for the uptake of the widest range of substrates by *E. coli* vesicles, Kaback has suggested that D-lactate oxidation plays a special role in active transport. It has also been observed that inhibitors of D-lactate oxidation fall into two classes with respect to their effect on active transport; if once a transported substrate is accumulated, an inhibitor is added, one class promotes efflux but

the other class does not. According to Kaback, the first class acts after a site of energy coupling, and the second class acts before this site.

One possible explanation for these differences between the behaviour of vesicles and that expected from Fig. 1 is that energy is diverted into the uptake of the electron donors themselves; other possible explanations have been discussed in numerous articles (for review, see Kaback 1974). But, whatever the correct explanation, it is clearly necessary to resolve these differences. Our view is that experiments such as those to be described bear out the predictions of the chemiosmotic theory and are difficult to reconcile with concepts of a discrete site of energy coupling and of a special role for D-lactate oxidation.

Association of proton movements with sugar transport

A central feature of the chemiosmotic theory is that the uptake of even neutral sugars should be accompanied by the uptake of protons. This was elegantly demonstrated by West (1970) and by West & Mitchell (1972, 1973) for the transport of β-galactosides by *E. coli*. That this is by no means a special case is shown by experiments conducted in our laboratory with other sugars and their non-metabolizable analogues. Since the experimental methods used for such demonstrations are rather unusual, we propose to discuss one such experiment in detail.

The strain of *E. coli* used (ML 35) is *lac y*: it was chosen for study of uptake of D-galactose and of its non-metabolizable analogue D-fucose because the absence of lactose transport eliminates a possible source of ambiguity. The organisms were grown on glycerol in the presence of D-fucose to induce systems for galactose transport (Ganesan & Rotman 1966).

After being harvested, the cells were suspended in buffer in the absence of nutrient at 37 °C for one hour to deplete them of endogenous energy supplies, washed once more and made up into a stock suspension of 35 mg dry mass/ml. This stock suspension was diluted 14-fold into lightly-buffered medium maintained in strictly anaerobic conditions, and the pH of this suspension was recorded continuously. When the recording was steady, galactose was added. An immediate alkaline pH change was observed, which rapidly reversed as the galactose was metabolized (Fig. 3). When the experiment was repeated, but D-fucose was added instead of galactose, the immediate alkaline pH change was again observed but now was not reversed. Since D-fucose is a substrate for galactose transport system(s) but is not metabolized, this result shows that the movement inwards of D-fucose is accompanied by the movement inwards of protons (which, of course, cannot be experimentally distinguished from the outward movement of hydroxy ions). The addition to these cells of isopropyl

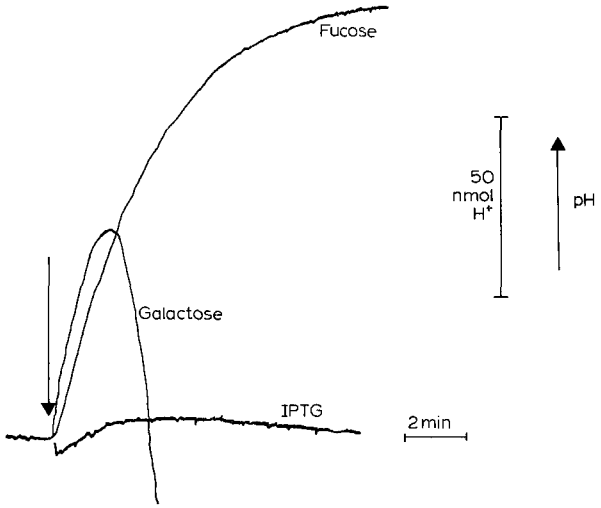

FIG. 3. Changes in pH after the addition of sugars to a *lac y* strain of *E. coli* induced for galactose uptake. Three separate experiments are shown, in which 25 μmol of the indicated sugars were added to a suspension (7 ml) of *E.coli* ML 35 at the point indicated by the downward arrow. (For conditions see the text.)

β-D-thiogalactoside (IPTG), a substrate for the β-galactoside transport system, did not elicit proton movements. This contrasts with the observation (Henderson 1975) that IPTG does promote effective uptake of protons if, in identical conditions, it is added to Lac y^+ strains of *E. coli* that have been induced for lactose uptake, and confirms that, in strain ML 35, the lactose carrier played no part in the movement of protons associated with the uptake of fucose or galactose.

Similar experiments performed with a variety of sugars and of strains of *E. coli* have confirmed that sugar uptake is linked to that of protons, and that this association exhibits the specificities shown by the transport proteins (see pp. 250–254). Thus, as shown in Table 1, suspensions of the Hfr-Cavalli strain of *E. coli*, when induced with D-fucose for galactose transport, took up protons at the same time as they took up D-galactose and D-fucose but, in contrast, L-fucose (which is not a substrate for the galactose transport systems) did not promote this proton movement nor did L-arabinose. Whereas the uptake of methyl β-D-thiogalactoside (a substrate for the β-galactoside uptake system) was accompanied by the uptake of protons, albeit to a much lesser extent than seen with the substrates of the galactose uptake systems, no proton movements were detected when methyl β-D-galactoside was used. This

TABLE 1

Effective H^+ uptake into fucose-induced *E. coli*

Addition	*Initial rate of H^+ uptake (nmol mg^{-1} min^{-1})*	*Total H^+ uptake (nmol mg^{-1})*
D-Fucose	1.8	4.7
D-Galactose	1.6	3.7
Methyl β-D-thiogalactoside	0.3	1.0
Methyl β-D-galactoside	0.0	0.0
L-Arabinose	0.0	0.0
L-Fucose	0.0	0.0

The measurements were made with conditions similar to those for Fig. 2, differing only in the addition of 10 μmol of the sugars to an anaerobic suspension of volume 5.5 ml containing 17 mg dry mass of bacteria.

is particularly interesting since the uptake of this substrate has been used as one criterion to characterize one type of galactose transport system, specified by the *mglP* gene which also specifies a 'binding protein' (Boos 1972). The significance of this observation remains to be established. With *E. coli* strains induced for arabinose uptake, the medium became alkaline during inward movement of arabinose (Henderson 1975; Henderson & Skinner 1974). Both the specificity for transport substrates and the specificity for inducers showed that the arabinose transport system characterized by Englesberg and co-workers (Novotny & Englesberg 1966; Hogg & Englesberg 1969) was involved, and not arabinose movement on the β-galactoside or galactose carriers.

If the change to a more alkaline medium observed during translocation of lactose, galactose, or arabinose is a transmembrane pH gradient, its appearance should be prevented by prior incubation of the cells with uncoupling agents. Experiments have been reported that confirm this prediction (West & Mitchell 1973; Henderson 1975; Henderson & Skinner 1974).

If the changes in pH are to be observed, respiration must be abolished by the removal of oxygen and the cells must be depleted of endogenous energy reserves. If this were not done, then according to the scheme of Fig. 1, proton movement inwards on the sugar uptake systems would be followed by movement outwards through respiration or ATP hydrolysis, and no net pH changes could be observed. Measurements in these stringent conditions do not necessarily provide evidence for the operation of proton translocation during active transport in 'normal' conditions. However, cells used for experiments such as those of Fig. 3 and Table 1 appear to be undamaged by this treatment: there is no loss of viability and, when incubated with substrates in aerobic conditions,

the organisms are not impaired in their ability to effect active transport and to maintain considerable concentration gradients of sugars.

'*Uncoupled*' *mutants*

Wilson *et al.* (1972) isolated mutant strains of *E. coli* in which energy could no longer be transduced into transport of β-galactosides. It was subsequently shown (West & Wilson 1973) that the simultaneous uptake of β-galactosides and of protons was seriously impaired in these mutants. The coincident loss of both these functions implies that the proton movements are indeed the *modus operandi* of the energy input. In one of the strains the lesion mapped in the *y* gene (West & Wilson 1973) indicating that the M protein was altered in its ability to respond to a pH gradient.

Involvement of an electrical potential

The evidence described above indicates that a transmembrane pH gradient, that is a difference in chemical potential of protons on either side of the membrane, can be coupled to transport of sugars. The chemiosmotic theory states that a transmembrane *electrical* potential, alone or in combination with a pH gradient, can also be coupled to transport (Mitchell 1963, 1966, 1970). This can be tested experimentally because it is possible to induce transmembrane electrical potentials in organisms or in subcellular vesicles susceptible to the antibiotic valinomycin; when K^+ ions are present inside but not initially outside, addition of valinomycin promotes electrogenic efflux of K^+, which thus creates an electrical potential (Henderson 1971; Harold 1972). If the transport of sugars is coupled to an electrical potential, K^+ efflux should drive the influx of sugar. This prediction has been confirmed for lactose transport by vesicles of *E. coli* (Hirata *et al.* 1973; Kaback 1974) and by intact cells of *Streptococcus lactis* (Kashket & Wilson 1973).

CONCLUDING REMARKS

We have considered the coupling of biological energy to a vectorial process in *Escherichia coli* for two main reasons. First, the well characterized system of genetic exchange possessed by the K12 strain has made it particularly suited to the isolation of mutants in which the active transport process can be studied in isolation from interference by other metabolic reactions. Secondly, the organism's catholicity of diet and its ability to grow either in the presence or absence of oxygen have particularly facilitated study of the control and ener-

getics of transport activity. Advantage has been taken of these two properties to obtain much information on the phenomena of active carbohydrate transport—indeed, not only were 'permeases' recognized and named thus through work with *E. coli* (Rickenberg *et al.* 1956) but much of our knowledge of their functioning stems from work with this organism and with subcellular vesicles derived from it. We are aware of the danger in applying interpretations derived from *E. coli* uncritically to other organisms. However, extensive studies (reviewed by Harold 1972) with the strict anaerobe, *Streptococcus faecalis* (which lacks a respiratory chain and hence cannot carry out oxidative phosphorylation), accord with the chemiosmotic view of active transport and support a unitary concept of energy transducing mechanisms.

It is apparent, from the type of questions we have asked, that the study of active transport is still in the largely descriptive phase. It is still necessary to determine what happens and when, and it is not yet possible to discuss with any degree of rigour how it happens. We realize that there are observations apparently at variance with the model round which we have built our discussion (Fig. 1), and that this model does not provide an understanding of the molecular mechanism of translocation, but we believe that neglect of the chemiosmotic theory upon which it is based will make it even harder ultimately to achieve such an understanding.

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Discussion

McGlashan: What is the Mitchell hypothesis?

Kornberg: The behaviour of mitochondria and of chloroplasts (Klingenberg pp. 23–40, and Whatley, pp. 41–63) provides striking evidence for the spatial orientation of electron carriers in the membranes. Mitchell, in 1961, recognized that this arrangement could provide an explanation of energy transduction alternative to the chemical hypotheses which then held the field. The main tenet of his hypothesis is that these respiratory components are so organized that one proton is translocated across the coupling membrane, from the inside of mitochondria or bacteria to the outside, every time that two electrons pass within the membrane from one carrier to the next. It is also necessary to assume that the energy-transducing membrane is impermeable to protons and to other ions, except through specific systems that Mitchell calls 'porters'. Oxidative phosphorylation would then be explained by a passage inwards of the protons (down the proton gradient established as a result of respiration) across the porter that is also an ATPase: this is postulated to shift the equilibrium of the ATPase reaction in favour of synthesis of ATP from ADP and inorganic phosphate. The transport of sugars, likewise, can be viewed as the simultaneous passage of protons and of the sugar across the membrane through the appropriate porter system, the proton gradient again providing the energy for this process.

Huxley: The hypothesis originated in connection with the mechanism of oxidative phosphorylation. A longstanding difficulty, which Professor Klingenberg mentioned, was that chemists had failed to identify intermediates that might be regarded as providing the coupling between the oxidations through the cytochrome chain and the phosphorylation of ADP to ATP. Originally Mitchell envisaged the coupling not through an identifiable chemical substance but through the generation of a potential difference between the inside and the outside of the mitochondrion and also through a difference in hydrogen ion concentration. The synthesis of ATP is immediately driven by these gradients rather than by the existence of some chemical compound.

Hastings: The attempts to isolate a concentration gradient as a chemical entity appear futile in retrospect. One could enlarge on this explanation by writing the equation for electron transport as alternating between a two-electron carrier and a one-electron carrier located on opposite sides of the membrane showing the appearance of hydrogen ions in the equation.

Klingenberg: No. That is one formulation, but it no longer holds for the cytochrome chains. I have pointed out that there may be a shift of the pK when electron transfer occurs through an electron carrier and, therefore, the changes of the pK may affect translocation of the hydrogen ions although the species undergoing oxidative reduction does not have to be a hydrogen carrier.

McGlashan: Can this potential difference be detected and, if so, how?

Kornberg: Yes. Direct measurement of an electrical difference, with an electrode, is out of the question for mitochondria. We can, however, measure a difference in hydrogen ion concentration.

Huxley: But that is separate from an electrical potential difference.

Klingenberg: We can determine the membrane potential from the gradient of rubidium ions (see p. 107) in the presence of specific ionophores such as valinomycin.

Huxley: The method is to introduce into the membrane a molecule which makes the membrane permeable to Rb^+, for example. The Rb^+ ions will distribute themselves according to the Nernst equation for the potential which exists.

Another technique is that used by Witt for chloroplasts for which this same question of the mechanism of phosphorylation arises. Witt observes that the spectrum of some carotenoids is altered by the membrane potential difference.

McClare: Witt (1974) applied a potential between two aqueous phases separated by a phospholipid bilayer (BLM) which contained carotene. He then followed the change in the spectrum of the carotene as a function of potential; it changed in the same way that it does in a chloroplast during

photosynthesis. In this way he measured the potential change across the chloroplast membrane.

Keynes: Is the evidence from fluorescent probes (and in a sense this is what the carotenoid experiment is) accepted as demonstrating the occurrence of change of the gradient?

Whatley: The experiments only reveal a change in the environment of the probe which may be related to a change in gradient, whether an electrical field or an ion concentration. If one imagines that when the probe is 'activated' it is in the membrane and when it is not 'activated' it is out of the membrane, one clearly cannot say anything directly about the electrical gradient.

Glynn: In connection with the transient changes in pH you observed during galactose uptake (p. 256), it may be worth mentioning the striking effect accompanying sugar uptake in *Neurospora* (Slayman & Slayman 1974). In that organism, the membrane potential can be measured. When an added sugar, even a non-metabolizable sugar, was being taken up, the membrane was transiently depolarized; when all the sugar had been taken up the potential returned to its initial value.

Kornberg: Dr Roseman himself showed that *Neurospora* took up sugars by a *non*-phosphotransferase system (Neville *et al.* 1971).

Huxley: This is a resurrection of a story that was current many years ago about secretion of hydrogen ions in the stomach and other kinds of active transport for which a direct link with the electron transport chain was postulated (see Conway 1953). Then, the evidence against this seemed to mount steadily. For example, when Hodgkin and others injected ATP into squid axons, the transport system was re-energized—it appeared to be driven by ATP. This longstanding theory has now found a substrate for itself!

Kornberg: In the work on gastric secretion done more than twenty years ago by R. E. Davies lies the most dramatic evidence for the dissociation of water; protons are abstracted from water so that (as the advertisement puts it) 'the acid in your stomach will burn a hole in the carpet'. Moreover, as a dramatic demonstration, Dr Davies used to show that frog gastric mucosae, connected in series, can power small electric light bulbs.

Roseman: I should like to be the devil's advocate on the entire issue of proton-motive force driving transport of solutes. First, let me acknowledge the many remarkable contributions made by Mitchell. Not only did he propose this concept and the role of protons in oxidative phosphorylation and photophosphorylation, but he has made many other key suggestions as well. For example, he suggested that membrane-bound enzymes were responsible for transport and that conformational changes in these proteins could explain the process. (It is a pity that he did not coin the term allostery.) Nevertheless, I

have yet to see convincing evidence that the sole and prime source of energy required for the active accumulation of solutes is by co-transport with protons. The difficulty in the theory is that two components are involved in proton-motive force, the membrane potential and the pH gradient, and these parameters are invoked when the stoichiometry or kinetics do not meet the requirements of the theory. In some published papers, only one of these parameters is measured and the other is assumed. In others, explanations are offered which are basically rationalizations, such as proton leaks. In any case, clear and unequivocal evidence for a single system has not yet, to my knowledge, been presented.

Kornberg: Kaback, who argued most forcibly against it, has now withdrawn many of his objections.

Roseman: No one doubts proton movement; the problem is what is the driving force in solute transport? I favour Dr Glynn's ideas; clearly, phosphorylated proteins transport non-metabolizable solutes, such as sodium, potassium and calcium ions. Protons may be necessary to generate phosphoprotein; Dr Glynn described the use of a sodium gradient to synthesize ATP.

Kornberg: Sugar uptake coupled to cation transport can be demonstrated in vesicles, which are totally unable to phosphorylate oxidatively. Similarly, intact bacteria transport sugars and also take up protons when suspended in arsenate buffer.

Roseman: I realize that, but cells poisoned by arsenate still contain high-energy phosphates, including ATP—admittedly at low concentrations, but still there. The evidence can be argued both ways. I believe that it will finally all come together, the proton movement and phosphoprotein entirely by analogy with the sodium–potassium system and the calcium pump. They are not in discord nor do I imagine that Mitchell would say that they were. We should not rule out the old established model simply by invoking proton motive force.

Glynn: I am not clear why people are so ready to accept that ATP synthesis may be driven by a downhill movement of sodium ions or of calcium ions and yet dislike the idea that it may be driven by a downhill movement of hydrogen ions. In the Mitchell hypothesis, the electron-transport chain pumps hydrogen ions across the membrane one way and then the hydrogen ions run down the electrical or concentration gradient the other way, synthesizing ATP.

Kornberg: Nobody disputes that. The point at issue is that made by Professor Klingenberg, namely, are we thinking of ATP hydrolysis as a necessary component of the transport process, or are we saying that the proposed high-energy state can both drive the reaction between ADP and P_i

to give ATP and also drive transport? These processes are reversibly linked. If we change the system so that, for example, transport is forced, then we should be able to force ATP synthesis.

Glynn: But wasn't it shown years ago that calcium accumulation in mitochondria could be driven either by ATP, when it was oligomycin-sensitive, or by electron transport, when it was unaffected by oligomycin?

Kornberg: Those experiments are open to other interpretations: the original one is now doubtful. Dr Roseman is saying that transport necessarily and obligatorily involves phosphorylation of proteins. I am saying that it doesn't.

Roseman: No. I am saying that I leave the question open, but that we should not disregard the clear evidence for existing systems. The movement of protons may give rise to phosphoproteins; these phosphoproteins may be labile and hard to detect but may act in the processes under discussion.

Huxley: What we want is evidence. The perennial question is, to what extent can we argue from one system to another? Nature is full of surprises when things turn out not to be the same.

Glynn: It seems uneconomical for a cell to have to phosphorylate the same sugar twice. Do cells use the phosphotransferase system when the phosphorylated product is not toxic, and the system in which sugar is released to the cell interior as free sugar only when the accumulation of sugar phosphate would be harmful?

Kornberg: The system Dr Roseman described has adapted to taking up glucose and immediately feeding it into the metabolic machinery of the cell *as glucose 6-phosphate*, which is the one compound that can enter that pathway directly and is, therefore, immediately utilized. It never accumulates. The artificial glucose analogue, methyl α-glucoside, can also be phosphorylated by the glucose-specific Enzyme II (see Roseman, p. 234), but the methyl α-glucoside thus formed is not metabolized further. Hence, *now* a toxic sugar phosphate does accumulate and the organism then stops growing.

Roseman: Sugar phosphates are generally toxic at high concentrations in bacteria.

Huxley: What is the driving force of the accumulation of gluconate in ghosts or vesicles in the presence of lactate? Is the lactate being oxidized?

Kornberg: The driving process in some systems is the removal of electrons from D-lactate (this process has been extensively reviewed by Kaback [1972]), although, with gluconate, it appears to be from L-lactate. In the course of this reaction, the lactate is oxidized to pyruvate.

Huxley: Do the vesicles have mitochondrial-type enzymes within the membrane?

Kornberg: Yes; they contain a flavin-linked 'lactate oxidase' system which feeds electrons into cytochrome, which goes to oxygen.

Huxley: So, you have something that will pump hydrogen ions.

Kornberg: Anaerobically, one must provide an alternative electron acceptor. Kaback has shown that fumarate or nitrate, for example, is suitable for that purpose.

Hess: Does such a system allow the uptake of amino acids?

Kornberg: The initial studies with vesicles were on the uptake of proline (Kaback 1972); so, the same kind of system should work. Indeed, W. A. Hamilton and his colleagues in Aberdeen and F. M. Harold in Denver have shown that even strictly anaerobic bacteria will accumulate amino acids in response to electrical gradients.

Taylor: How is the 300 mV membrane potential related to the hydrogen ion concentration?

Klingenberg: In the second version of this hypothesis, Mitchell proposed that the movement of protons was electrogenic, so that a net proton concentration gradient need not be built up in order to generate this high membrane potential difference. However, the accompanying movement of anions can break down the membrane potential and build a pH gradient. The choice of ΔpH against membrane potential depends on the system: for instance, chloroplasts build a steep pH gradient because chloride ions move in compensatorily; the mitochondrial membrane apparently cannot do this—it shows a small pH gradient and, therefore, a high membrane potential. In both cases, the potential contributions are generated by the electrogenic movement of protons.

McGlashan: What do you mean by electrogenic?

Klingenberg: It means that the proton movement is not compensated by a negative charge movement.

Kornberg: This is why I used the phrase a proton and/or electrical gradient.

Huxley: This term electrogenic originated in connection with sodium pumps. The pump actively extrudes sodium at the same time actively taking up potassium. If it works on a 1:1 basis there is no net transfer of charge and the system is termed not electrogenic, but it does create concentration differences and may produce a membrane potential secondarily by raising the internal concentration of potassium. Then, if there are other independent channels through the membrane by which potassium can diffuse out, that will secondarily produce a membrane potential. In that case, the pump itself would be said not to be electrogenic. However, the experimental evidence indicates that in many cases the number of sodium ions extruded per second exceeds the number of potassium ions taken up. In addition to altering the concentrations, a procedure which will indirectly affect membrane potential, the pump action directly

creates a net outward current. That is described as electrogenic. Closely related systems may or may not directly create a current across the membrane by net movement of charge.

Glynn: More direct evidence supports electrogenicity than just the stoichiometry.

Huxley: Yes, one often finds that the membrane potential has been driven far beyond the equilibrium potential of any of the ions present.

Hess: The principal barrier to an understanding of a mechanism of coupling between a diffusion gradient as a driving force and the generation of chemical bonds, such as in the synthesis of ATP, lies in the fact that such a thermodynamic view does not provide a mechanistic answer to the problem. Only definition of the chemical species, such as a phosphorylated intermediate being protein-bound, or similar possibilities will lead to understanding.

Huxley: Nobody has yet mentioned the very effective synthesis of ATP that can be obtained with a calcium gradient in the calcium-uptake vesicles from muscle, which have been known for several years (Makinose & Hasselbach 1971). Fragmentation of muscle yields a preparation of membrane vesicles derived from an intracellular system. These vesicles actively take up calcium from the surrounding solution if the ATP is present—this is the normal function of the structures from which these are derived in the muscle. The calcium ions in the main part of the cell contents are removed into these vesicles and this movement causes relaxation. In these isolated vesicles, it is possible to produce a calcium gradient in which the diffusion of calcium down the concentration gradient causes synthesis of ATP from ADP and P_i strongly against the free-energy difference calculated from the concentrations. The phenomenon appears more striking here than in red cell ghosts.

Kornberg: Largely through the work of Gibson & Cox (1973) *E. coli* mutants are available that are devoid of ATPase activity. Such mutants will grow aerobically or anaerobically on substrates that allow ATP synthesis by glycolysis (or by other substrate-linked phosphorylations). They can also carry out active transport, but only in aerobic and not in anaerobic conditions. This must mean that the energy generated by respiration can be coupled to active transport even if (as in these mutants) no ATP synthesis occurs. However, in anaerobic conditions, it is the hydrolysis of ATP (which cannot occur in the mutants) that normally generates the high-energy state required for transport.

Taylor: In the calcium system, we know the intermediates. The calcium gradient is real. Yet I have the feeling that in all these arguments the hydrogen ion gradient is virtual.

Huxley: Do you mean it is impossible to measure it independently?

Glynn: But only if there is no electric potential.

Huxley: Presumably, it is the electrochemical potential difference of hydrogen ions on the two sides that is relevant to the synthesis. The energy difference ΔE/mol H^+ is given in equation (1), where $\Delta\psi$ is the potential

$$\Delta E = RT\Delta\ln[H^+] + F\Delta\psi \quad (1)$$

difference across the membrane. Depending on the situation, either $RT\Delta\ln[H^+]$ or $F\Delta\psi$ will dominate, but what will drive this synthesis of ATP is the sum of the two terms.

Taylor: That is the reason why the hypothesis is so difficult to accept because the effect cancels out. The calcium gradient is really there and readily measureable.

Kornberg: If you feel that the hydrogen ion gradient is virtual, what do you think we are measuring?

Taylor: In the calcium system, which is better understood, the ratio of Ca^{2+} ions inside to outside is maybe 10^4. Simple arithmetic convinces one that synthesis of ATP can proceed. However, in your arguments, the large gradient has disappeared by compensating it with something else: the pH difference is not 4.

Kornberg: The actual pH gradient we observe *in our experiments* will depend upon the relative buffering capacities of the internal and external environments, as well as the number of protons transported.

Huxley: You are measuring it as a pH change in the external solution. You, very reasonably, deduce that the opposite happens inside, although you haven't actually measured the internal pH. With the calcium systems, it is possible to centrifuge the vesicles down and then measure their calcium content.

Gutfreund: Couldn't this be satisfied also by a protein inside taking up a proton? The protein could then change its conformation and then pass into the 'high-energy' state.

Huxley: Conformation changes are always a way out!

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The origin of force in skeletal muscle

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Abstract Since the proposal and rapid acceptance of the sliding-filament theory in 1953–1954, numerous suggestions have been made for the cause of the sliding movement. When the amount of overlap is varied by altering the initial length, the maximum tension is directly proportional to, but the speed of shortening under zero load is independent of, the amount of overlap. This suggests strongly that a relative force between thick and thin filaments is produced by independent force-generators distributed within each overlap zone. These force-generators are identified with projections (cross-bridges) on the thick filament, each consisting of part of a myosin molecule. Measurements of the 'tension transients' when the length of a stimulated muscle fibre is suddenly altered show that the range of action of each cross-bridge is 10–15 nm. The travel within a single contraction may be many times greater, so each cross-bridge must act cyclically by attaching, exerting a force and detaching. Details of the tension transients suggest that each cross-bridge makes its movement in two or three steps, each with a potential energy change a few times kT. Each cross-bridge contains also an elastic element in series. It is sufficient, on present evidence, to postulate that the only action of ATP is to dissociate the cross-bridge from the thin filament after it has completed its stroke.

From early this century until about 20 years ago, it was generally supposed that active muscle contained long continuous filaments of 'myosin' (Kühne 1864) or 'actomyosin' (Szent-Györgyi 1943), which tended to shorten under the influence of one or other of the metabolic reactions that take place when a muscle is stimulated. Engelhardt & Lyubimova (1939) showed that myosin is itself an ATPase, confirming the idea that the splitting of ATP is the reaction which is most directly coupled to the shortening and which supplies the energy that appears as heat and work during contraction. Even within this framework, however, there was plenty of room for controversy. For example, it was not known whether the splitting of ATP took place during a contraction, or whether the shortening was in some sense a spontaneous event which was reversed by

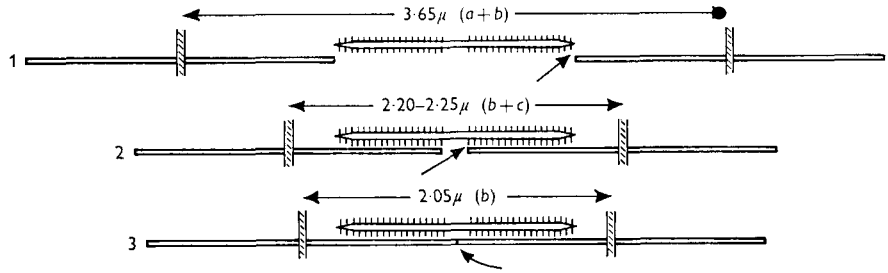

FIG. 1. Diagram showing various degrees of overlap in the sliding-filament array of frog striated muscle. At the centre, a single thick (myosin) filament, and at each side, a thin-filament complex (principally actin, with Z line at the middle). The diagram is based on the arrangement of filaments found by H. E. Huxley (1953) and the measurements of Page & H. E. Huxley (1963): *a*, length of thick filament (1.60 μm); *b*, length of thin-filament complex (2.05 μm; now believed to be somewhat less); *c*, length of segment without projections at centre of thick filament (0.15–0.20 μm). Stages of overlap: (1) just zero overlap; (2) just complete overlap of projections by thin filaments; (3) thin filaments meet at centre of sarcomere.

Overlap increases linearly with decrease of sarcomere length from stage 1 to stage 2, but does not alter between stage 2 and stage 3, where mechanical interference with shortening begins. (From Gordon *et al.* 1966*b*, by permission of the *Journal of Physiology*.)

ATP splitting during the relaxation phase. Again, much discussion ranged over the question of whether the tendency to shorten arose from the formation of new bonds in the shortened condition, as for example in the 'internal energy' theories of Meyer (1929), Buchthal & Kaiser (1951) and Polissar (1952), or whether it was attributable to an increased freedom of the molecular chains to fold or coil under the influence of thermal agitation, as in the 'entropy theories' of Wöhlisch (1940), Pryor (1950) and Morales & Botts (1952).

SLIDING FILAMENTS

All thinking about the contractile process had to be redirected when it was found in 1953–1954 that the length changes in striated muscle of vertebrates, whether active or passive, take place by a relative sliding movement of two interdigitating sets of filaments in each half-sarcomere and not by length changes *within* any of the major filamentous structures in the muscle fibre. This is summarized in the familiar diagrams of Fig. 1. There is no need to repeat the evidence for the sliding process (see for example Hanson & H. E. Huxley 1955; A. F. Huxley 1957; H. E. Huxley 1960, 1966) or to discuss the extent to which it had been foreshadowed in forgotten work of the nineteenth century (see A. F. Huxley 1957, 1969).

People often speak of the 'sliding-filament theory of muscular contraction', but I regard this as a misnomer. The fact that the length of myofibrils changes

by sliding, with little or no change of length in the individual filaments, is now well established, and is no longer in the category of a 'theory'; on the other side, the sliding-filament idea by itself leaves completely open the question of what makes the filaments slide and, therefore, is not a *theory of contraction.* Indeed, so far from providing a single 'theory of contraction', the idea of sliding filaments opened up numerous fresh possibilities of molecular mechanisms for the coupling of chemical change to shortening, and a great number of proposals have been made in print. Elsewhere (A. F. Huxley 1974), I have divided these published theories into eight categories; only two of these categories have been directly excluded by experiment, leaving a wide range of ingenious hypotheses that still must be regarded as possibilities—most are entirely speculative. I propose to discuss some experimental results that lead to definite suggestions about certain features of the mechanism. I hope that these will be of interest to those whose direct concern is with energy transduction systems other than muscle.

As a start, it is fortunate that there are observations pointing fairly definitely toward one broad class of theories, namely, those which postulate a series of independent force-generators between adjacent thick and thin filaments within each overlap zone. This idea was put forward explicitly by A. F. Huxley & Niedergerke (1954) and was one of the possibilities considered at the same time by H. E. Huxley & Hanson (1954).

EVIDENCE THAT THE CROSS-BRIDGES ARE INDEPENDENT FORCE-GENERATORS

The first piece of evidence for the existence of independent force-generators comes from the manner in which the tension developed by a striated muscle fibre depends on the length at which it is held and, therefore, on the amount of overlap of the two types of filament. The well known measurements of Ramsey & Street (1940) had already shown that, in frog muscle fibres, the active tension developed during tetanic stimulation is a maximum if the fibre is held at its slack length (striation spacing s about 2.05 μm), and declines almost linearly as the length is increased, reaching zero at about double the slack length ($s \approx 4.1$ μm). Qualitatively, this is to be expected if tension is proportional to the length of each overlap zone, and we pointed out (A. F. Huxley & Niedergerke 1954) that this observation suggested that the total tension in each filament is the sum of contributions from a number of sites spaced at regular intervals within each overlap zone; if each site contributes an equal amount of relative force between thick and thin filaments, the total force on each filament will be proportional to the number of contributing sites, and therefore to the length of the overlap zone.

However, measurements with the electron microscope showed that the thick-filament length is about 1.5–1.6 μm and the thin-filament length (including the filaments on both sides of one Z line: Fig. 1) is about 2.0 μm. Hence, overlap and therefore tension development should drop to zero when s = 3.5–3.6 μm—substantially less than the value suggested by the measurements of Ramsey & Street. However, the discrepancy is due to a trivial circumstance: the sarcomeres near each end of a fibre do not elongate as much as those in the middle when a fibre is stretched (A. F. Huxley & Peachey 1961). Active contraction occurred only in sarcomeres whose length was below a critical value which lay between 3.5 and 3.6 μm, i.e. in adequate agreement with the sum of the filament lengths obtained by electron microscopy. Later, we built a device which holds constant the length of a selected segment of an isolated muscle fibre, chosen so that the striation spacing within it was practically uniform (Gordon *et al.* 1966*a*,*b*). With this device we again found a linear decline of active tension as the fibre length was increased, but now the length at which active tension approached zero agreed satisfactorily with that at which overlap ceases to exist. Another feature that we found is that active tension is practically constant for sarcomere lengths between about 2.0 and 2.2 μm, the linear decline with length beginning at the latter value. The decline at lengths shorter than 2.0 μm appears to be a complicated phenomenon, due partly to interference between the filaments as shortening proceeds (Gordon *et al.* 1966*b*) and partly to incomplete activation of the contractile material (Taylor & Rüdel 1970; Rüdel & Taylor 1971).

From the early days of sliding filaments it had seemed that material connections between thick and thin filaments must exist during contraction and in the rigor state (Hanson & H. E. Huxley 1955, p. 251; A. F. Huxley 1957). The existence of such 'cross-bridges' was demonstrated by electron microscopy in insect flight muscle by H. E. Huxley & Hanson (1957) and in vertebrate muscle by H. E. Huxley (1957) who showed further that they consisted of lateral projections from the thick filaments which appeared to attach themselves to the thin filaments. He also showed (1963) that a segment about 0.2 μm long at the middle of each thick filament is free from these projections. These features are shown diagrammatically in Fig. 1. It follows that at all striation spacings greater than 2.0 μm, the active tension found by Gordon *et al.* is closely proportional to the number of projections that are overlapped by thin filaments: the plateau of tension between 2.0 and 2.2 μm corresponds to lengths where all the projections are overlapped, since each thin filament extends beyond the end of the region of the thick filament that carries projections.

On this evidence, therefore, it becomes extremely probable that (*a*) total

tension in each filament is the sum of contributions from sites distributed in each overlap zone and (*b*) these sites are to be identified with the projections seen with the electron microscope on the thick filaments.

The second type of observation that suggests independent force-generators is the relation between overlap and speed of shortening. If the passive resistance to shortening is negligible, then under zero external load the maximum speed of shortening should be attained even with few cross-bridges in action: if there is no force to be overcome, additional bridges will not help the shortening process. In 1954 we had no observations on shortening speed at different degrees of stretch comparable to those of Ramsey & Street on isometric tension. However, there were long-standing observations (Jasper & Pezard 1934) showing that speed of contraction varies inversely with sarcomere length when different muscles of a single arthropod species are compared (all striated muscle fibres of vertebrates have nearly the same sarcomere length). If the sliding speed in any one half-sarcomere were the same in long-sarcomere and short-sarcomere muscles, the overall shortening speed per cm length of fibre would be proportional to the number of overlap zones in series, and hence inversely proportional to sarcomere length. This we cited (A. F. Huxley & Niedergerke 1954) as further evidence for independent force-generators. More recently, the inverse relation between sarcomere length and shortening speed in arthropods has been confirmed by Atwood *et al.* (1965), and in my laboratory we found (A. F. Huxley & Julian 1964; Gordon *et al.* 1966*b*) that the speed of unloaded shortening in an isolated frog muscle fibre is practically constant as the sarcomeres shorten from a length of say 3.0 to 2.0 μm.

Since 1954, it has seemed to me that these two observations—tension proportional to overlap but speed independent of overlap—are sufficient grounds for concentrating our attention on the class of theories which postulate independent force-generators in each overlap zone, and the evidence mentioned on p. 274 implies that these force-generators are the cross-bridges seen with the electron microscope. At present, this point of view is widely though by no means universally accepted. In what follows, I shall confine myself to this class of theories.

FEATURES OF CROSS-BRIDGE ACTION

Numerous more or less detailed proposals for the nature and mode of action of the cross-bridges as force-generators have been made (Weber 1956, 1958; A. F. Huxley 1957; Tonomura *et al.* 1961; Davies 1963; H. E. Huxley 1969; Podolsky *et al.* 1969; A. F. Huxley & Simmons 1971*b*; McClare 1972).

Some general considerations must be taken account of in any theory of cross-bridge action:—

Cyclic operation. It is impossible to suppose that a cross-bridge remains attached throughout a contraction in which much shortening occurs. For example, if the sarcomere length shortens from 2.5 to 1.5 μm, the amount of relative motion between thick and thin filaments in any one half-sarcomere is 0.5 μm, whereas the *total* length of a myosin molecule—of which the cross-bridge presumably represents a part—is less than 0.2 μm. All the theories now under consideration assume for this reason that even during a single contraction a cross-bridge performs repeated cycles of attachment, pulling and detachment.

Detachment. Another point common to almost all these theories is that the detachment step is assumed to be a rapid consequence of interaction with a molecule of ATP. This is a natural assumption since 'actin' and 'myosin' were originally identified (Straub 1943) by a procedure in which the proteins were separated from their complex actomyosin by the action of ATP (Szent-Györgyi 1943), and this dissociation has since been shown by rapid-reaction techniques to be very fast (Lymn & Taylor 1971).

Range of action. A value of roughly 10 nm for the amount of shortening that a cross-bridge can produce before it has to detach was deduced by H. E. Huxley (1960) and by Davies (1963) from estimates of the tension per cross-bridge and the work that can be done per mole of ATP used. This order of magnitude is confirmed (A. F. Huxley & Simmons 1971*a*,*b*, 1973) by direct measurement of the amount of shortening that a frog fibre (near 0 °C) will take up within 1–2 ms, i.e. faster than the time presumably needed for cycles involving detachment and reattachment. This distance is two or three orders of magnitude too great to be caused by the process of formation of an ordinary chemical bond between a site rigidly fixed to the thick filament and another rigidly fixed to the thin filament. This was a real difficulty for theories such as that of Weber (1956, 1958) which included no feature that could spread out over a long distance the forces associated with the formation of chemical bonds; it was probably one of the reasons why several completely different theories (e.g. vernier theories, where this difficulty does not arise) were put forward between 1954 and 1970 (for references see A. F. Huxley 1974).

Of the independent-cross-bridge theories, some evade the difficulty by blandly postulating a sufficiently large 'conformational change' in the part of the myosin molecule which forms the cross-bridges. In at least three theories,

however, explicit proposals have been made for dealing with the problem:

(1) I put forward a scheme in 1957 in which the myosin site is on a side-piece attached by an elastic link to the rigid structure of the thick filament.

(2) In Davies' theory (1963) the cross-bridge is a part of the myosin molecule which is in an elongated random coil state while unattached but which, as a result of electrostatic interactions, is converted into a much shorter α-helical structure on attachment to a thin-filament site. The full range of movement is thus subdivided between the large number of hydrogen bonds which are formed during the shortening of the cross-bridge.

(3) In the theory of H. E. Huxley (1969) (see Fig. 5*a*) the force originates in a tendency for the head (subfragment 1, S-1) of the myosin molecule to rotate around the point where it attaches to the thin filament. The myosin head acts as a crank which transmits a force through the connecting rod S-2 to the main structure of the thick filament. The rotational movement of the crank converts small movements near the axis of rotation into much larger movements at the point where the connecting rod is attached.

One reason for incorporating an elastic element in the side-piece of my 1957 theory was that the quick-release experiments of A. V. Hill and others had suggested that active muscle contained an instantaneous elasticity that appeared to be in series with the contractile component, which was assumed to shorten at a speed that was a function only of the load. At that time there was no evidence that this elasticity resided in the cross-bridges themselves rather than in the filaments or perhaps the Z lines. Our recent experiments, with greatly improved time resolution, have confirmed the existence of an instantaneous elasticity, though it appears to be two or three times as stiff as the earlier experiments had suggested. Our experiments also show (A. F. Huxley & Simmons 1971*a*; A. F. Huxley 1974) that when the initial length of the fibre is changed, this stiffness varies almost exactly in direct proportion to the overlap of cross-bridges with thin filaments, thereby suggesting—just as in the case of the tension developed on stimulation—that the elastic elements are actually within the cross-bridges, while the filament structure is almost completely rigid.

Thus, we now have good grounds for retaining an elastic element within each cross-bridge, though it needs to be stiffer than assumed in my 1957 theory, and the recent quick-release experiments show that it does not account for the whole of the range of action of the cross-bridge (see below, p. 280).

TENSION TRANSIENTS

In addition to showing that each cross-bridge contains a straightforward instantaneous elastic element, our recent records of the time course of the

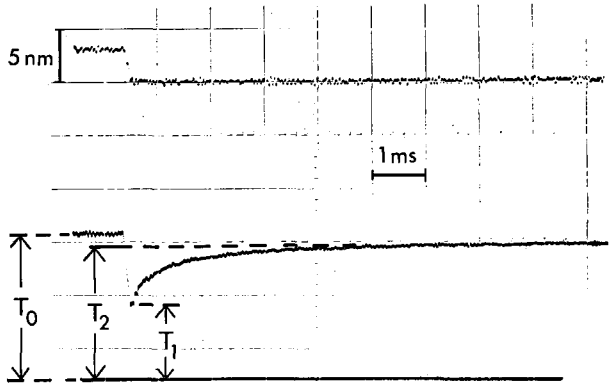

FIG. 2. Simultaneous records of length (upper trace) and tension (middle trace) in an isolated frog muscle fibre which is made to undergo sudden shortening during the plateau of an isometric tetanus. Bottom trace: baseline for tension. The amount of shortening is 3.0 nm in each overlap zone; the step is complete in 0.2 ms. Temperature 1 °C.

T_0 is the active tension before the step. As the length changes, the tension falls to T_1. It recovers in a few milliseconds to T_2 which is only a little less than T_0; the later stages of recovery toward T_0 are much slower (half complete in about 70 ms). The ratios T_1/T_0 and T_2/T_0 are plotted in Fig. 3 as functions of the size of the shortening step. (From unpublished experiments by L. E. Ford, A. F. Huxley & R. M. Simmons.)

tension change when the length of a stimulated muscle fibre is suddenly reduced have shown two interesting non-linear features during the first few milliseconds after the step change of length (A. F. Huxley & Simmons 1971*b*, 1973; A. F. Huxley 1974). In this period, the tension drop that occurs simultaneously with the shortening is partly reversed (Fig. 2). This behaviour is similar to that of a Voigt element (a spring in series with a parallel combination of spring and dashpot) but both the extent and the rate constant of the tension recovery vary in highly non-linear ways with the size of the shortening step (see Figs. 3 and 4).

Two types of explanation have so far been offered for these phenomena. According to Podolsky and his colleagues (Podolsky *et al.* 1969; Podolsky & Nolan 1973) the early tension recovery is attributed to rapid attachment of cross-bridges that had previously been free. We assume, however, that the recovery is due to changes in the state of cross-bridges that were already attached (A. F. Huxley & Simmons 1971*b*). We have now obtained strong evidence in favour of our view, since we find that the stiffness of the fibre—nearly proportional to number of attached bridges—is no greater after the early recovery phase than before the step (Ford *et al.* 1974). Detachment and reattachment of cross-bridges are, therefore, almost entirely confined to the later phases of slow recovery of tension toward the original value.

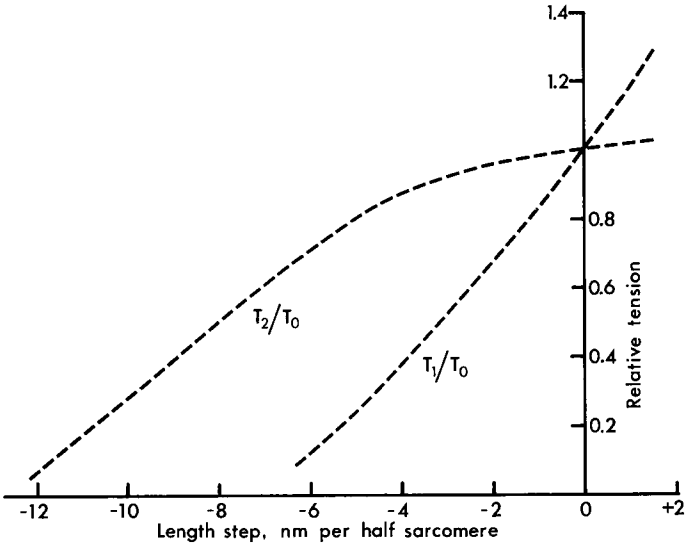

FIG. 3. Tension levels reached during transient responses such as that shown in Fig. 2: T_1, extreme tension reached during the step change of length; T_2, tension approached during the early recovery phase. Both expressed as fractions of T_0, the active tension measured before the step change of length (see Fig. 2 for definitions of T_0, T_1 and T_2). (From the same experiment as Fig. 2.)

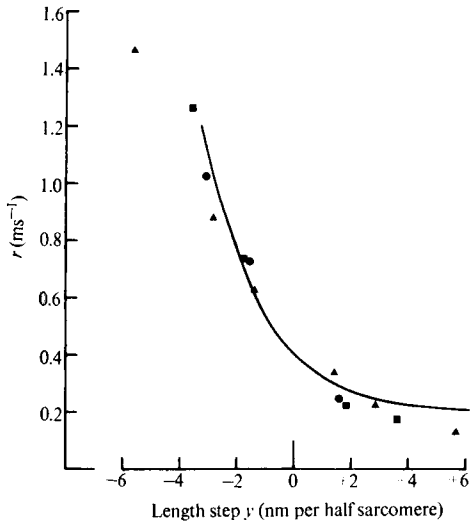

FIG. 4. Rate constant r of quick recovery phase after a length step of magnitude y (positive for stretch). Measured on records similar to Fig. 2 but with less sharp step change of length (step complete in about 1.0 ms). (From A. F. Huxley & Simmons 1971*b*, by permission of *Nature [Lond.]*.)

Hence, the nearly complete tension recovery in the first few milliseconds after a release of, say, 3 nm in each overlap zone (Fig. 2) implies that the elastic element in each cross-bridge is re-stretched by a fairly rapid change in some other component of the cross-bridge that is mechanically in series with it. The fact that the tension recovery becomes progressively less complete as the size of the shortening step is increased (Fig. 3) shows that the amount of length change that can be taken up by this second element in the cross-bridge is limited (to about 6 nm). Evidently, this element is the part of the system that can be regarded as the actual origin of the muscular force: after a small release, it re-stretches the elastic element that transfers the force to the filaments. Presumably, its action likewise stretches the elastic element when a cross-bridge first attaches itself to the thin filament, so that its total range of action is 10–15 nm.

FURTHER ANALYSIS OF CROSS-BRIDGE ACTION

My discussion so far shows that there are good experimental grounds for the following propositions about the origin of force in striated muscle:—

(1) The total force on any one filament is made up of contributions from each cross-bridge that is within the zone of overlap of thick and thin filaments.

(2) The cross-bridges act more or less independently of one another.

(3) Each cross-bridge acts in a repeated cycle of attaching to the thin filament, exerting force, and detaching.

(4) Each cross-bridge contains two elements in series, one of which behaves with a passive instantaneous elasticity while the other can do net mechanical work, maintaining tension as the filaments slide past each other for a distance of the order of 10 nm.

These propositions are extremely specific in comparison with the enormous range of mechanisms that might cause two sets of filaments to slide relative to one another. They provide a definite framework for our thinking about the questions in which this meeting is interested, but they do not in themselves constitute an answer to those questions. They do suggest that it is useful to think separately about two groups of events within the whole process of production of force, namely, (1) the attachment and detachment of cross-bridges and (2) the process that does mechanical work while the cross-bridge is attached.

Strong hints about both these aspects come from experimental observations that I have not yet mentioned.

Attachment and detachment of cross-bridges

In order to fit the relations between force, speed of shortening and rate of heat production, found by A. V. Hill (1938), I put the following features into my 1957 theory:—

(1) The rate constant for attachment of a bridge is 'moderate' (about 30 s^{-1} in frog muscle at 0 °C);

(2) The rate constant for detachment is 'low' if the filaments are prevented from sliding relative to one another;

(3) The rate constant for detachment (with splitting of ATP) becomes 'high' (order of 200 s^{-1}) when the filaments have slid about 10–15 nm (in the shortening direction) since the cross-bridge attached;

(4) If the fibre is stretched during contraction, the attachment process can be reversed without splitting of ATP.

I showed that relationships of these kinds could account more or less quantitatively for the rates of shortening and of energy liberation as they were known in 1957. More recent work has modified, for example, the dependence of heat rate on speed of shortening (Hill 1964), and has raised doubts about the relations between chemical change and the 'maintenance heat' and 'shortening heat' of Hill's analysis (Aubert & Lebacq 1971; Gilbert *et al.* 1971; Dickinson & Woledge 1973; Curtin *et al.* 1974). When these problems are resolved, it will no doubt be necessary to revise the details of our assumptions about the kinetics of attachment and detachment of cross-bridges, but I expect that the four points mentioned above will remain valid as a first approximation. The kind of modification that it seems may be needed is, for example, a two-stage instead of a single-stage attachment (A. F. Huxley 1973) and, possibly, detachment without ATP splitting when shortening has carried the cross-bridge far beyond the position at which its tension drops to zero (Podolsky & Nolan 1973, p. 663).

There can be little doubt that one aspect of the coupling between chemical and mechanical events lies in this dependence of rate of ATP splitting on the position of the attachment of a cross-bridge to the thin filament relative to the origin of the cross-bridge on the thick filament. I shall discuss later (p. 284) the question of whether this may be the only point in the cycle where an interaction with ATP is directly involved.

Events while a cross-bridge is attached

The key question remains: what are the events by which a cross-bridge develops a force after it has attached and maintains it in spite of moderate

amounts of shortening (Fig. 3)? In my 1957 theory, I postulated that no separate event was needed: the cross-bridge attached only when the elastic element happened to be stretched by Brownian movement, because an enzymic site needed for the attachment process was located in the right position on the thick filament. It is now clear that this idea is too simple, because it does not account for the transients that have been recorded in recent years by applying a step change of load (Podolsky 1960; Civan & Podolsky 1966) or of length (A. F. Huxley & Simmons 1970, 1971*a*,*b*, 1973). I have already mentioned that the non-linearity of the T_2 curve in Fig. 3 indicates that each cross-bridge contains an active element in series with its elastic element. The other non-linear feature in the tension transients is that the speed of the early tension recovery becomes progressively greater as the size of the shortening step is increased (Fig. 4). We showed (A. F. Huxley & Simmons 1971*b*) that this phenomenon can be explained simply if the 'active' element shortens stepwise, moving from one to the next of a series of perhaps three stable positions with progressively lower potential energy (intervals of a few times kT). We gave a simplified mathematical treatment in our article; a qualitative explanation of the way in which the system produces the variation of rate constant with step size is as follows. When the length of the fibre is held constant so that the filaments cannot slide, each cross-bridge divides its time between two (or perhaps more) of its stable positions, the potential energy differences between them tending to make it move forward (in the direction which stretches the elastic element and causes shortening) and the tension in the elastic element tending to make it move back. When a small amount of shortening (sliding of filaments) is imposed suddenly, this equilibrium is disturbed because the tension in the elastic element is reduced. The rate with which the new equilibrium is approached is governed by the activation energies for the step movements in the two directions. Each of these contains a component representing the energy needed to displace the cross-bridge out of the stable position in which it has been. For a backward movement, this is the whole of the activation energy since the elastic element acts in the direction of helping the bridge to move to its new position, so that no further work needs to be done. In the case of a forward motion, however, the work in stretching the elastic element appears as a second component in the activation energy: the lower the tension, the smaller is this component, and therefore the higher the rate constant for the forward stepping movement. In a shortening step the tension is reduced in proportion to the size of the step, hence the forward rate constant gets greater as the size of the step is increased.

The only other explanation for this variation of the rate constant that has yet been proposed is that of Podolsky *et al.* (1969) and Podolsky & Nolan

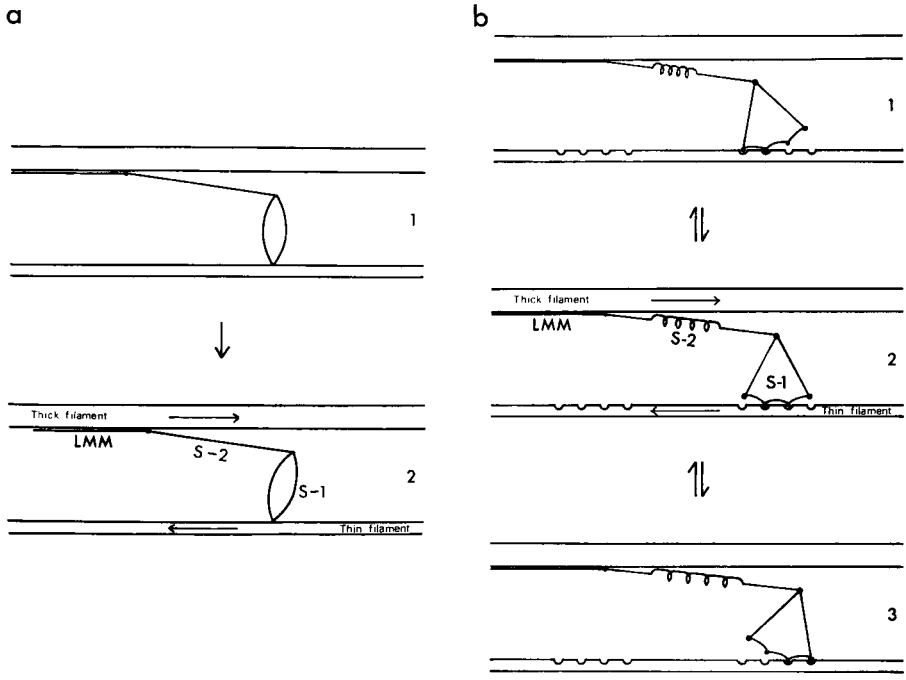

FIG. 5. Diagrams of cross-bridge structure and action: *a* (adapted from H. E. Huxley 1969), the myosin head (S-1) tends to rotate abouts its point of attachment to the thin filament, exerting a force on the thick filament through the S-2 portion of the myosin molecule acting as an inextensible link. (*b*) A proposal (A. F. Huxley & Simmons 1971*b*), incorporating the stepping element and the elastic element, suggested by the tension transient experiments, into a structure based on *a*. (From A. F. Huxley 1974, by permission of the *Journal of Physiology*.)

(1973), but as has already been mentioned, their theory requires an increase in stiffness after the step and this is not found (Ford *et al.* 1974). Our theory certainly cannot be regarded as established at the present time, but we feel that it has a good chance of turning out to be substantially correct.

Structures responsible for the elastic and stepping components

Numerous possibilities are open (A. F. Huxley & Simmons 1973; A. F. Huxley 1974) for the structural identification of these two elements in a cross-bridge. The one that we used for illustrative purposes (A. F. Huxley & Simmons 1971*b*) is shown in Fig. 5*b*; it is one of several ways in which the elements inferred from our kinetic experiments can be fitted to the structural arrangement proposed by H. E. Huxley (1969) on the basis of X-ray diffraction

and electron microscopy. I still regard it as an attractive possibility but it must not be thought that there is evidence for preferring it to, for example, a system in which the tip of the myosin head is rigidly attached to the thin filament and the stepwise movement occurs by bending at a point or points within the myosin head itself (Fig. 12*a* of A. F. Huxley 1974).

Nature of coupling between chemical and mechanical change

An interesting distinction between these two schemes is relevant also to other ways in which the stepping process might occur. In the system shown in Fig. 5*b*, the *only* way in which ATP needs to intervene is by causing the cross-bridge to detach after it has reached position 3: it can then reattach in position 1 at a site further along the thin filament and it is ready to drive the sliding motion by progressing again to position 2 and position 3. If, however, the stepping process is completely *within* the myosin head, then ATP must not only detach the cross-bridge but also reverse the stepwise change. Conceivably, the splitting of one molecule of ATP could drive both of these events; another possibility suggested by Tonomura *et al.* (1961) is that two ATP molecules are used per cycle, one to detach the cross-bridge and the other to reverse the driving movement of the cross-bridge.

There is again no firm evidence on which to decide between these two kinds of possibility. A result which can be taken as favouring the idea that ATP has only a single action is that of Mannherz *et al.* (1974), referred to by Gutfreund & Trentham (pp. 69–81). This result shows that almost the whole of the energy drop in the enzymic splitting of ATP by myosin occurs at a single step out of the seven or so stages that have been identified in that process.

McClare (1971) has claimed that all theories based on cyclic attachment and detachment of cross-bridges, involving only the formation and breakage of ordinary chemical bonds, are excluded because they contradict the second law of thermodynamics, and that it is only possible to make plausible theories of this kind by disregarding back-reactions. All the theories I have been discussing in this paper are in the class which McClare attacks, but I am nevertheless confident that they are not invalid in the way he suggests. T. L. Hill (1974) has recently worked through an example with full treatment of back-reactions and has shown that net work is done in the cycle, thus directly disproving McClare's case.

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Discussion

Hastings: I always imagined that H. E. Huxley's model (Fig. 5*a*) contained universal joints.

Huxley: That view depends on whether you consider that the myosin head (S-1) has to be able to attach to every actin along the spiral structure of the thin filament or whether actin molecules to which the myosin head can attach are only occasionally pointing in the right direction. This consideration differs from the question how it produces force. He believes, and so do I, that rotation about the attachment is one-dimensional. We can consider the joints at the ends of S-2 to be universal joints. The novelty that he introduced was the placing of the force generation at the point where the myosin attaches to the thin filament (see p. 277)—I don't think he considers that as a universal joint.

Hastings: No; but it can rest in any orientation because of the universal joints that lead up to it. The physical relationship between the two elements is not obliged to be altered during movement.

Huxley: Another feature of this scheme is that the hinges at both ends of the S-2 element allow the system to be almost independent of the separation between the thin filaments. Although our evidence that tension is proportional to overlap is *prima facie* acceptable, it only becomes so if we assume that force per site is the same whatever the overlap, and as we change the length the lateral spacing changes very considerably. Although this still needs to be finally explained, H. E. Huxley's suggestion was the first satisfactory basis for tension per site being independent of lateral spacing.

E. Krebs: It is hard to visualize the next cycle.

Huxley: We must then incorporate elements of the 1957 theory (A. F. Huxley 1957): when the cross-bridge reaches its final position, ATP can attack it and cause dissociation in the same way as in solution. One feature of such a system is that the only thing that ATP has to do is to detach it, so making it available to attach at the beginning of the next cycle (p. 254). One can think of many

other models that incorporate a spring and a stepping process, but most of them need to be rewound by ATP before the next cycle.

E. Krebs: In your model, the spring has collapsed before the next cycle.

Huxley: Yes, then it is stretched by this tendency for the stepping to progress. We must assume that, with suitable enzyme specificity, the head attaches so as to be poised for forward movement (position 1 in Fig. 5*b*). If it attached in the final position (position 3), the mechanism would be a total failure. To postulate that the enzymic situation is such that attachment to position 1 proceeds with a high rate constant in contrast to low rate constants for attachment to the other two positions is not unreasonable.

Keynes: Where does calcium fit into this model?

Huxley: One calcium ion attaches to the troponin which influences the tropomyosin (which is all on the thin filament). However, I don't incorporate calcium in this model; the explanation of the action of calcium will present the same problem whatever specific model we propose. We still have to understand how changes within the thin filament affect tension generation. It is still controversial whether simply the number of attachment sites is altered or whether there is a change in the force–velocity relation. Whether the attachment all goes onto one actin monomer of the thin filament or whether part of the attachment is to one monomer and another part to adjacent monomers, nobody knows. Another unsolved question is whether calcium acts also on the myosin side. However, I have made no attempt to incorporate explanations for these in this model.

McGlashan: Do you know the sequence of potential energies as the head rocks from position 1 to 3?

Huxley: The potential energy levels in the sequence are separated by a few multiples of kT (see p. 282), but at present we have no good evidence about how many steps compose the sequence. Three positions with two steps seem perfectly possible. With fewer steps and, therefore, bigger potential energy steps, the theoretical relation between rate constant and force in the spring becomes steeper. From the steepness of this relation, we can make our estimate of the size of the steps. A problem lies in the fact that different sites in the overlap zone differ relatively in position; the spacings on the myosin filament differ from those on the actin filament. We measure an average of sites which are displaced relative to one another without knowing over what range to average. This introduces ambiguity about how much allowance we should make for this averaging in putting a value on the real variation of the rate constant if the cross-bridges were all in the same position relative to their actin sites. This is one unfortunate feature of the theory.

McGlashan: What might cause this difference of a few kT per 'rock'?

Huxley: This depends on the chemistry of the site. If the attachment is electrostatic (and the great sensitivity of the attachment to ionic strength implies the importance of an electrostatic interaction), the answer would be in how close the charges on the sites can approach each other and how far they are held apart by hydrophobic structures.

Weis-Fogh: Is the linking peptide chain between the part of myosin which is attached to the rod, and the head, a kinetically-free chain?

Huxley: The chain is capable of melting and extending, according to Burke *et al.* (1973). It is normally highly α-helical. Most protein chemists say that it is not likely that S-2 could act as a spring, but I do not regard this question as settled.

The most solid aspect of my theory seems to be that each cross-bridge contains an elastic element. This might not depend on the extension of a long structure but might rather be a rotary elasticity at the point of attachment. Our experiments provide no evidence on this. We need evidence about the position and fluctuation of these heads which might be derived from X-ray diffraction or from the experiments of, for example, Nihei *et al.* (1974) who, by labelling the head with fluorescent compounds, find that its attitude changes. If this movement has components at frequencies of a few thousand Hz, such experiments might tell us directly what is moving. The X-ray data indicate a gross movement of the tropomyosin molecule which, in the absence of calcium, just gets in the way of the myosin head coming down to the monomers.

Gutfreund: Are there consequently any mechanical changes in the properties of the muscle?

Huxley: That is hard to find out since it is difficult to measure the cross-bridge properties in a resting muscle. Using laser-light scattering, Ishiwata & Fujime (1972) claim to have seen changes in the stiffness of the thin filament on addition of calcium, but Carlson & Fraser (1974) using the same technique are not convinced. Certainly, that is an approach which gives evidence about the flexibility of separated thin filaments.

Lipmann: Do you think that the connection really separates and finds a new point of attachment? Is it possible that in position 3, the head collapses fan-like (2 in Fig. 1 overleaf) so that when the 'fan' opens again (3) the head is at the beginning of the stepping process and connection is retained?

Huxley: One of the points of the attachment can lose the load, because many others in parallel with it still bear the load. The number of theories that can be produced that incorporate these features of a few steps of energy levels plus a spring is almost unlimited. I am afraid people will latch on to one diagram believing it to be supported by evidence; hence my emphasis on alternative structural possibilities (A. F. Huxley 1974). Fig. 5 was principally designed to illustrate the energy relations.

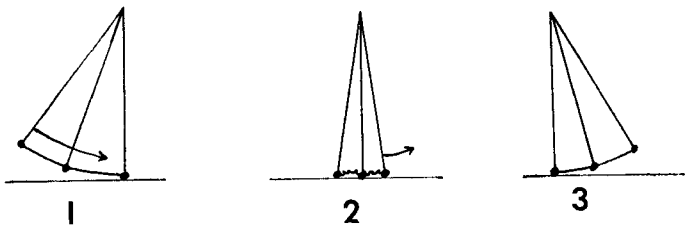

FIG. 1 (Lipmann). Hypothetical scheme for the collapse of the myosin head.

Wilkie: Can your new theory go hand in hand with your 1957 theory?

Huxley: Yes. We suppose that, at the end of the stroke, the myosin head is in an attitude which somehow allows ATP to dissociate it and then is able to attach again. This provides one of the essential features of the 1957 theory which was that the rate of detachment only reaches high values after the working stroke has been completed. We then must postulate that the rate constant for attachment is only a fraction of the high rate constant of detachment when it is in the final position. The 1957 theory will look after attachment and detachment so that the overall rates of turnover are as found experimentally; the new theory describes what happens during the period while it is attached.

Weis-Fogh: Surely, one important difference is that in 1957 the movements of the cross-links were postulated to be caused by thermal agitation and not by stereochemical changes.

Huxley: Yes. In the recent proposals, stretching of the elastic element by thermal agitation is not necessary; any action due to it is of minor importance, because most of the work is done by the stepping forward. The natural extension of this is to suppose that all the work originates thus. Previously, I held this rather implausible notion that thermal motion caused the element to get into the position where it attached, extending the spring, and that nothing more happened. That theory fails to explain the transients.

Roseman: Can the N^6-amino groups of the lysines in the proteins be acetylated? If so, does that stop the whole process?

Taylor: I don't think that experiment has been done though myosin has been variously modified, including *N*-acetylated. But nobody has done that experiment and measured the actomyosin binding constants at the same time. However, we have measured the rate of formation of the actin–myosin complex as a function of ionic strength and the results show that electrostatic interactions are important.

SPASMONEMES, A NEW CONTRACTILE SYSTEM

Weis-Fogh: As we are discussing the transfer of chemical energy into mechanical work, particularly in muscle, I should emphasize that there appear to be three basically different types of motile systems known at present: (1) the mechanism responsible for the movements of bacterial flagella; there is growing evidence that the flagellum is actively rotated at its base and that ATP is not the immediate source of energy. (2) The huge world of actively sliding filaments such as are found in muscle, in cells engaged in amoeboid movements, in plant cells with streaming cytoplasm (cyclosis), in actively beating cilia and flagella from plants, animals and unicellular organisms; although the filaments and their enzyme properties differ, the basic mechanism is an active sliding for which the immediate source of energy apparently derives from the hydrolysis of ATP or related compounds. (3) A new type of motile system, at present best known in vorticellid ciliates, that is, the contractile bands or rods formerly named myonemes but better called spasmonemes because they differ basically in structure and mechanism from muscle and the other systems listed above (Weis-Fogh & Amos 1972).

History

In some early theories of muscular contraction, the myofibrils were assumed to be relatively simple rubber-like structures which change from one state to another under the influence of metabolites or ions (cf. Karrer 1933; Pryor 1950). The discovery of sliding filaments naturally disposed of these theories and today they are mostly forgotten. However, some observations and experiments indicated that the myoneme in the contractile stalk of *Vorticella* and *Charchesium* behaves differently from other motile systems and more in accordance with the simple idea. Thus, Schmidt (1940) observed that the birefringence of the extended myoneme is high and that it is considerably reduced during shortening, in contrast to muscle. Levine (1956) found that glycerinated stalks contract when some bivalent ions are added and extend when EDTA is admitted. Hoffmann-Berling (1958) working in Hans H. Weber's laboratory obtained the greatest effect with calcium and strontium ions in small concentrations, also finding that the extended or contracted state can be maintained indefinitely at the appropriate free calcium concentrations and that ATP does not seem to be involved. Using EGTA buffers, Amos (1971) found that after the intracellular membranes have been destroyed with detergents, the glycerinated stalks remain contracted when the solution contains 1 μmol/l free Ca^{2+} and extended in 10 nmol/l; the threshold is 0.5 μmol/l and is independent of the absence or

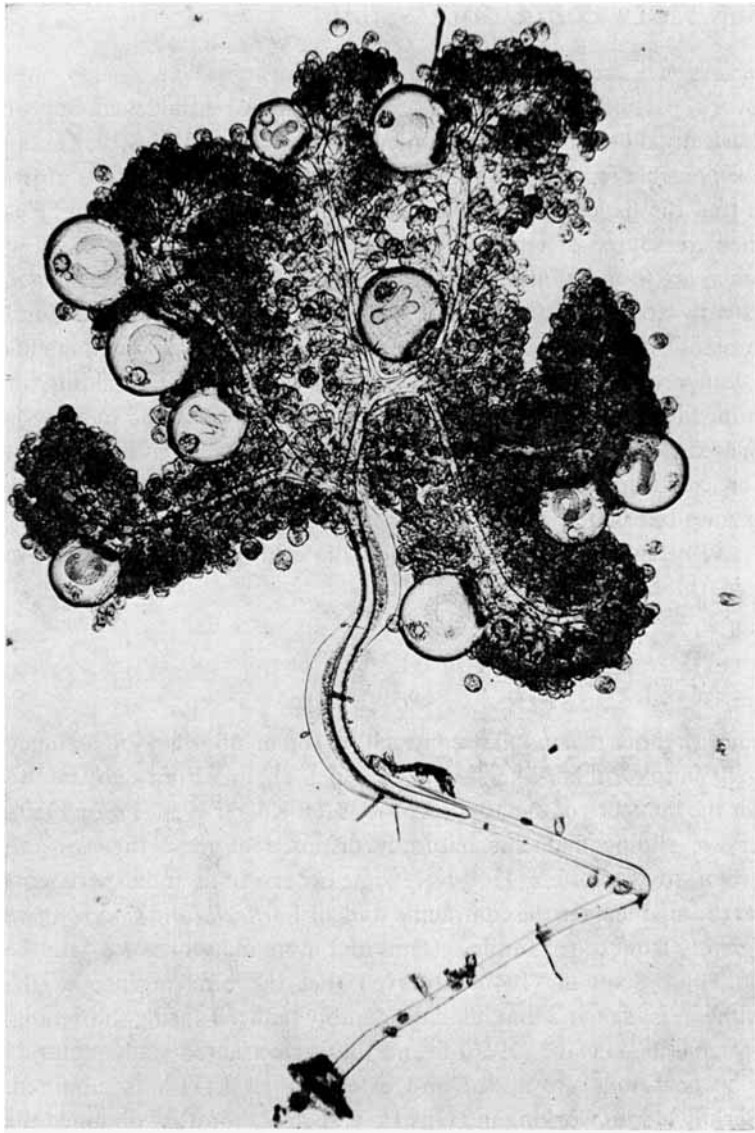

FIG. 1 (Weis-Fogh). A colony of *Zoothamnium geniculatum* with several hundred small cells (zooids) connected by contractile stalks to the contractile branches that unite into the common contractile stem in which the refractile spasmoneme is clearly visible. The cytoplasmic extension which surrounds the spasmoneme is seen as the grey envelope.

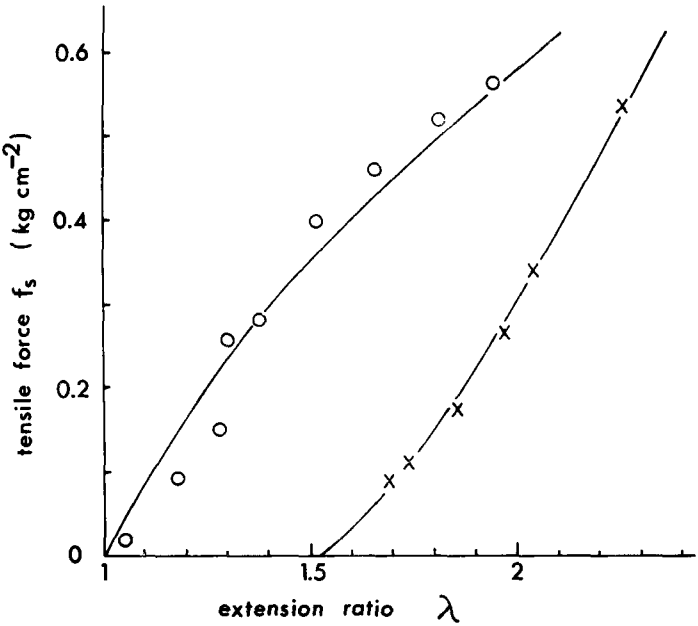

FIG. 2 (Weis-Fogh). Force–extension curves for an isolated spasmoneme at high (○; 10 μmol/l) and low (×; 10 nmol/l) calcium ion concentrations (from Weis-Fogh & Amos 1972). The line drawn for the results at higher concentration is the theoretical curve for an ideal kinetic rubber.

presence of Mg^{2+} at least up to 25 mmol/l. He also estimated the work done by a naturally contracting spasmoneme: the energy can be accounted for by the change in chemical activity of free calcium when the concentration increases from 0.01 to 1 μmol/l and the amount of calcium ions liberated (probably from the extensive tubular system of vesicles inside the spasmoneme) corresponds to 1.1 mmol in a volume equal to that of the spasmoneme itself. Earlier claims (Townes & Brown 1965) that ATP influences these results were not confirmed but more direct studies were not possible until a giant spasmoneme became available.

Properties of giant spasmoneme

Amos & I have recently found a colonial vorticellid *Zoothamnium geniculatum* (Fig. 1) which has a common contractile stalk with a uniform spasmoneme about 1 mm long and 30 μm in diameter in large specimens (Weis-Fogh & Amos 1972). The spasmoneme can be pulled out from the stalk as a giant cell organelle, either before or after glycerination, and suspended from a micro-

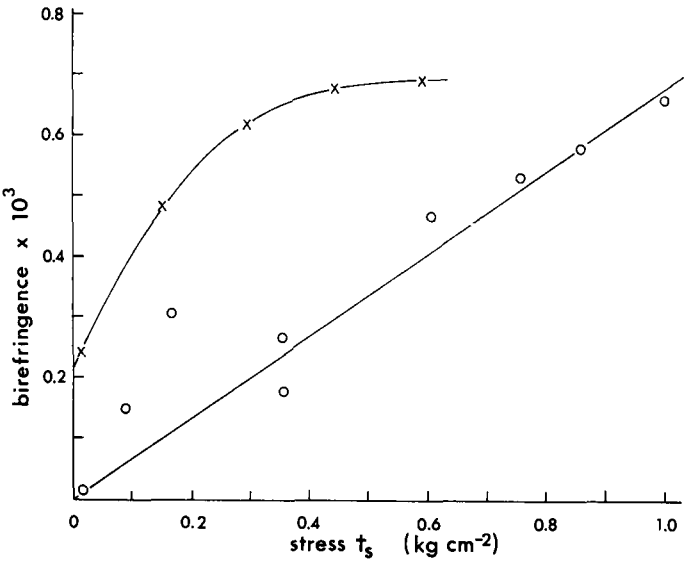

FIG. 3 (Weis-Fogh). Changes in birefringence with stress of the same spasmoneme as in Fig. 2 (from Weis-Fogh & Amos 1972): ○, 10μM-Ca^{2+}; ×, 10nM-Ca^{2+}.

balance for mechanical and optical investigations. Both at high (10 μmol/l Ca^{2+}) and at low calcium concentrations (10 nmol/l), Fig. 2 shows that the spasmoneme behaves as a rubber-like body with a low elastic modulus of about 6×10^4 N m^{-2}. It can be extended reversibly up to four times its unstrained length but otherwise there is a marked difference between the properties in the two solutions. In the higher concentration of free calcium ions the organelle behaves as a perfect rubber, and the line drawn is the theoretical curve for an ideal kinetic rubber $f = G\, v^{1/3}\, (\lambda - \lambda^{-2})$, where f is the force/unstrained area, G is the elastic modulus, $v\,(= 0.15)$ is the volume fraction of dry proteins in the swollen spasmoneme, and λ is the extension ratio. In accordance with this, the birefringence in Fig. 3 (circles) increases linearly with stress and is zero when the sample is not strained.

When the calcium concentration is lowered to 10 nmol/l, the unstrained spasmoneme extends spontaneously by more than 50% (Fig. 2, ×) and becomes birefringent (Fig. 3, ×). In other words, the originally isotropic network becomes anisotropic mechanically and optically and a new and steeper force–extension curve now results. High speed cinematography of living colonies has shown that, although small changes in swelling are likely to occur, the natural contraction is not accompanied by any marked change in swelling (Weis-Fogh & Amos, unpublished results), and the same seems to apply to the isolated

preparation in Figs. 2 and 3. The spasmoneme, therefore, becomes both longer and more rigid when calcium is removed.

The two curves in Fig. 2 may resemble the length–tension diagrams of a resting and contracted muscle. However, they represent true functions of state in the thermodynamic sense and differ, therefore, fundamentally from the steady-state diagrams for other contractile systems which depend on continuous hydrolysis of ATP or other energy-delivering compounds. The spasmoneme curves only depend on the maintained concentration of free calcium ions. They serve as a reminder that even in biology nature sometimes works in a simple way.

As to the mechanisms, it is too early to be specific. It is possible but far from certain that the extension at low [Ca^{2+}] is caused by electrostatic repulsion between fixed negative charges and that addition of calcium ions may neutralize these charges and cause the network to collapse, as already suggested by Hoffmann-Berling (1958) and in agreement with early theories of contractility. However, so far, we have not been able to see any general effect of the ionic strength and the reaction is extremely specific to calcium and related ions; Mg^{2+} up to 25 mmol/l is unable to influence the threshold for the calcium response. Another possibility is that the linearly arranged protein molecules (Amos, unpublished results) contain binding sites specific to calcium ions and that the configuration of the molecules is changed by the presence or absence of free calcium. In both cases we must assume that the major length of the backbone consists of kinetically free chains. Otherwise, the rubber-like properties would be impossible. However, this does not exclude the possible presence of one or more specific regions the conformation of which is determined by Ca^{2+}. Only studies of the protein(s) themselves can give the answer.

Calcium-binding proteins

We have determined the absolute amount of calcium bound by the glycerinated spasmoneme in the contracted and extended states, using X-ray microprobe analysis (T. Weis-Fogh *et al.*, unpublished results). We placed the isolated spasmoneme in a drop of the appropriate solution on an aluminium-coated Nylon film, removed the surplus fluid, dried the sample and, finally, applied a top coating of aluminium to the preparation to increase thermal and electrical conductivity. The stage of the X-ray microprobe analyser (JEOL, JXA-50A) was kept below –120 °C throughout. Fig. 4 shows the transmission electron image at high contrast (too high to show the actual outlines) together with four scanning traces. The difference between the count rates (*b*) and (*c*) represents the true amount of Ca present in the spasmoneme; it closely follows

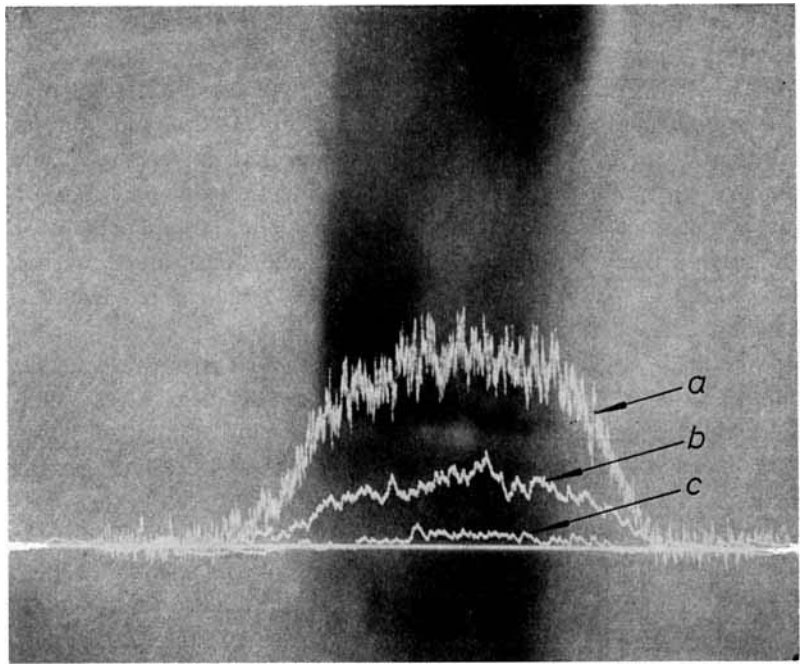

FIG. 4 (Weis-Fogh). X-ray signals from a spasmoneme (vertically oriented) when scanned by the electron beam in the transverse direction along the straight line, which is both the line of scanning and the base line for the other three traces: *(a)* the total mass present in the direction of the scan, measured by an energy-dispersive silicon detector; *(b)* the signal obtained by a diffracting spectrometer set at peak K_α-radiation for Ca; and *(c)* the off-peak signal of Ca.

the mass curve. The quantitative results, summarized in Fig. 5, were obtained by scanning rectangular areas in the middle of the spasmoneme. The *total* amount of calcium in the EGTA buffers was varied but the difference in *free* Ca^{2+} between the contracted and extended states was kept constant at 1 and 0.01 μmol/l, respectively. In this way, we could compensate for accretion effects and show that 1.7 g Ca/kg dry spasmoneme protein is bound during contraction. Since 40–60% of the organelle consists of two closely related proteins of molecular weight 20 000 (W. B. Amos *et al.*, unpublished results), this means that per molecule 1.4–2.1 calcium atoms (probably 2) are bound on contraction. In other words, the binding is very specific. Also, the affinity for calcium is extremely high. Even in a calcium-free solution containing 20mM-EGTA at pH 7, a small amount of calcium is retained, as seen in Fig. 5.

Recently, W. B. Amos *et al.* (unpublished results) have successfully isolated and characterized the two main protein components in the spasmoneme. Both

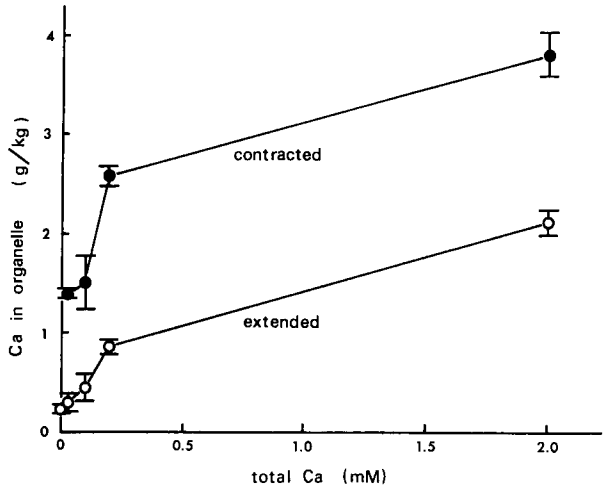

FIG. 5 (Weis-Fogh). The total calcium content of contracted and extended spasmonemes and its variation with the total calcium concentration in the EGTA (ethylene glycol bis-[2-aminoethylether] tetraacetic acid) buffers used; the *free* calcium ion concentration is 1 μmol/l (●) or 10 nmol/l (○) throughout the entire range.

are relatively small molecules (mol. wt. 20000), have very similar amino acid compositions, are acidic (pH 4.7–4.8) and are rich in aspartic acid and serine. They contain only a few aromatic residues and no cystine or methionine. As with troponin C, the spasmoneme proteins change their electrophoretic mobility when the free calcium concentration is raised from 10 nmol/l to 1 μmol/l; Mg^{2+} is without any effect in this respect up to 500 μmol/l. Amos *et al.* therefore conclude that the spasmoneme is composed largely of acidic proteins with molecular weights near 20 000 and with a high affinity for calcium, and propose that this class of proteins is named *spasmin.*

Eventually, it is from the structure and properties of spasmin as well as its arrangement in the intact structure that we shall discover how spasmonemes work.*

Hastings: Is it possible that a microcontraction cycle for muscle consists of one of these?

Weis-Fogh: I can only speculate. One can envisage a troponin component,

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which seems to push tropomyosin in and out of the groove, thereby controlling muscle contraction. It could have its mechanism explained in this way. Of course, in muscle, we are dealing with changes in calcium concentration of the same order of magnitude as in the spasmoneme system.

Huxley: The main shortening movement could not depend on calcium in this way because, for example, contractions continue in the absence of calcium if the troponin system is destroyed. You don't seem to be able to escape the conclusion that calcium is not necessary for contraction.

Hastings: But, a cycle based on the principle which has just been described could be constructed with a regulatory molecule other than calcium.

Weis-Fogh: The similarity between parvalbumin, troponin C and spasmin does indicate that calcium-binding proteins with some fundamental features in common are widespread and used in a variety of ways.

Wilkie: Is the calcium binding reversible in the sense that, if the *Vorticella* is stretched, the bound calcium drops off?

Weis-Fogh: That is unlikely, because the affinity for calcium is so high that it competes with the affinity for Ca^{2+} of EDTA at pH 7. These molecules must, to a large degree, be kinetically free chains, otherwise one would not observe the rubber-like behaviour. Specific sites must bind calcium and in doing so, the network becomes isotropic. When they release calcium, anisotropy is induced. However, in terms of energy per molecule, the changes are small.

Huxley: How long do these things survive?

Weis-Fogh: We have kept them and used them for months in the glycerinated state.

Wilkie: Since you are measuring calcium uptake and you can determine the chemical potential of the calcium, can you take this thing round a complete cycle and measure what fraction of the maximum theoretical work the system can deliver?

Weis-Fogh: We should be able to do this in the future. The system is so sensitive and reproducible in its reaction that we can use it for titrating calcium.

Gutfreund: Are there any general effects at high ionic strengths (higher than $1\mu M\text{-}Ca^{2+}$)?

Weis-Fogh: They are not specific. When we dramatically lower or increase the ionic strength, the mechanism still works.

Gutfreund: The response to an increase in ionic strength is interesting, because in the absence of Ca^{2+} the system may be kept in the extended state by electrostatic repulsion. The energy of the system, in a biological sense, is supplied by the removal of calcium. If there is electrostatic repulsion in the absence of calcium and one adds calcium, one can neutralize the charge. In the absence of calcium, the charges keep it apart.

Weis-Fogh: A simple electrostatic model of that kind will probably not hold because magnesium in physiological concentration up to 5 mmol/l has no effect. However, we still have many experiments to do when we have some more fresh material.

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How does ATP act as an energy source?

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Abstract Two misleading conclusions drawn from the classical treatment of thermodynamics are, first, that single molecules cannot be treated thermodynamically and, second, that all real processes must be irreversible. The first conclusion has meant that the disruptive effect of thermal motions has been ignored, the second has meant that dynamic forces which cannot exist without stored energy, have been left out of account. The effect of these beliefs is demonstrated by analysis in detail of a simple electrostatic model of muscle. It is shown that the necessity of protecting each step against decay of the stored energy, and the requirement that each should be specific, has the effect of stopping the model actually working. Moreover, the character of static forces, which are strongest after their stored energy has been used up, precludes their use in cyclic processes because as much stored energy is required to undo them later in the cycle.

Covalent intermediates are also examined from a quantum mechanical viewpoint and it is shown how these store energy which can be transferred by resonant transitions in the ground state when groups like phosphate are transferred chemically between molecules. But this analysis shows that, necessarily, the energy remains stored and therefore not available for muscular contraction. Then it follows naturally both that bond vibrations are produced in exothermic chemical reactions and that, because the force disappears when the energy has been used up, resonant exchange of stored energy in the excited state should solve the difficulties of previous approaches to these problems.

A muscle is a chemical machine. Yet it is also an assembly of molecular machines (McClare 1971) and is, therefore, very different from those chemical machines, like batteries, that we understand. For this reason it seemed valuable to take a close look at the discipline we use to study these machines—thermodynamics—to see whether this change to a molecular scale made any difference to the problem. The main conclusion drawn from this reappraisal was that an historical accident—namely, that 50 years elapsed after the second law was formulated before molecules were accepted as real—led to the mistaken

premise that thermodynamic equilibrium is *static*. This premise then led to two further misconceptions: (1) we cannot apply thermodynamic conclusions to single molecules and (2) only infinitely slow processes can be reversible. However, although it became clear later that every equilibrium is *dynamic* at the molecular level, these erroneous conclusions are still generally accepted. The logical paths by which these conclusions were reached, and the reasons why they are false, are given elsewhere (McClare 1971, 1974).

Stated baldly, these errors may seem simple enough to avoid, yet they are only too easy to make unconsciously for, unless we have a statement of the second law which explicitly applies not only at the macroscopic level but to molecular energies as well, it is impossible to know whether a particular muscle model is consistent with thermodynamic laws. This is why the urgent need for a new statement of the second law that is consistent with Brownian motion has been emphasized (Popper 1957; McClare 1971). In one possible solution to this problem we use the interval during which a machine performs a cycle, τ, to define a time-scale and then distinguish between stored energies (which last for longer than τ in a specific form) and thermal energies (which freely interchange and reach a Boltzmann distribution in a time less than τ). The second law then becomes 'Useful work can only be done when one form of *stored* energy is directly converted into another' (McClare 1971). But this means, if the above statement is acceptable, by proposing any model for ATP action that does not consider the lifetimes of all the intermediate forms of stored energy and how they become so stored, one is *in effect* making error (1), by not applying thermodynamic restrictions to the molecular model. The practical effect of error (2) is far more difficult to understand: it has been to unconsciously exclude from consideration a whole range of possible mechanisms (McClare 1971, 1972*a*).

Nowadays, experimental investigations are advancing so rapidly that the theoretical difficulties are highlighted even more clearly than in 1971 and it is possible to illustrate the argument more explicitly, for fewer possibilities remain. Although muscle will be used as the example, the same conclusions apply equally throughout bioenergetics.

THE ENERGETICS OF MUSCULAR CONTRACTION

The following summarizes those points which seem particularly important energetically: (i) ATP is the immediate energy-source (Cain & Davies 1962). (ii) The myosin head-group undergoes a *cyclic* process of attachment, tilting and detachment (Pringle 1967; Huxley 1971)—many times per muscle-twitch (Davies 1963); consequently, the external force applied to the thin filament is

not restraining the chemical reaction (McClare 1971). (iii) This important conclusion in (ii) is thoroughly reinforced by the observation that, even when a muscle is exerting its maximum tetanic tension, 80% of the head groups remain *unattached* to the thin filament (Huxley & Brown 1967; Eisenberg *et al.* 1972). (iv) The chemical kinetics also show that hydrolysis is independent of the actin: when MgATP is exposed to myosin (M) it first binds to form M·ATP, which then undergoes a conformational change to produce (M·ATP) (Bagshaw *et al.* 1975). This complex is rapidly hydrolysed ($k = 100\,s^{-1}$; Lymn & Taylor 1970) with the formation of a high energy complex ($M{\cdot}ADP/P_i$), which slowly changes ($k = 0.02\ s^{-1}$; Taylor *et al.* 1970) into a species from which the products can dissociate (see scheme 1). The high-energy

$$\begin{aligned} M + ATP \rightarrow M{\cdot}ATP \rightarrow (M{\cdot}ATP) \rightleftharpoons (M{\cdot}ADP/P_i) \rightarrow \\ M{\cdot}ADP{\cdot}P_i \rightleftharpoons M{\cdot}ADP + P_i \rightleftharpoons M + ADP + P_i \end{aligned} \quad (1)$$

character of ($M{\cdot}ADP/P_i$) is attested by two remarkable experiments: first, the complex changes back into (M·ATP) only ten times less rapidly than it is formed (Bagshaw & Trentham 1973), that is, the free energy of the hydrolysis at this step is only some 6 kJ/mol, and secondly, as the ($M{\cdot}ADP/P_i$) changes into $M{\cdot}ADP{\cdot}P_i$ (the low-energy product), internal energy is released over several minutes and can be measured calorimetrically as heat (Yamada *et al.* 1973). (v) In the presence of actin the first step is the *dissociation* of the actomyosin complex that forms spontaneously (Finlayson *et al.* 1969); the actin produced only combines *after* hydrolysis to increase the rate at which the high-energy complex releases energy (see schemes 2 and 3). Furthermore (vi),

$$A + M \rightarrow A{\cdot}M \overset{ATP}{\rightarrow} A + M{\cdot}ATP \rightarrow (M{\cdot}ATP) \rightleftharpoons (M{\cdot}ADP/P_i) \quad (2)$$

$$(M{\cdot}ADP/P_i) + A \rightarrow A{\cdot}M{\cdot}ADP{\cdot}P \rightleftharpoons A{\cdot}M{\cdot}ADP + P \rightleftharpoons A{\cdot}M + ADP + P_i \quad (3)$$

in view of the rapidity of the hydrolysis and the slowness of the decay of ($M{\cdot}ADP/P_i$) in the absence of actin, it may be that the myosin head-groups are in this high-energy state even *before* the nervous impulse arrives at a muscle in the 'resting' state. This proposal is made in a recent kinetic muscle model (Tawada *et al.* 1974). Finally, (vii) the observations of mechanical oscillation in both insect flight muscle (Pringle 1967) and in glycerol-extracted heart muscle (Steiger 1971) suggest that energy can be put straight into the ($M{\cdot}ADP/P_i$) state from outside the muscle.

Three further points should be made explicit since they are easily overlooked and yet are fundamentally important. First, the machine is so small that any temperature-differences must even out far more quickly than the head-groups can tilt, so that the cycle is isothermal. Secondly, the head-groups are continually

being bombarded as a result of thermal motions with average energy 1.3 kJ/mol ($\approx 2.2 \times 10^{-21}$J/molecule). Thirdly, the machine is immersed in water so that any kinetic energy possessed by a protein must be lost almost immediately. This last point is not generally appreciated (although by no means new) and is worth considering in detail.

THE EFFECT OF VISCOSITY ON MOLECULAR MOTIONS

Consider a spherical particle of radius r and density ϱ immersed in a fluid of viscosity η. Suppose that it is given an initial velocity of v_0 by some impulsive force. If the body is large compared with the molecules of the fluid (as is the case for proteins in water) then the frictional drag (F) is given by the Stokes–Einstein equation (4) (Einstein 1908). The drag is negative because it acts

$$F = -6\pi\eta r v \tag{4}$$

against the instantaneous velocity, v. The mass, m, of the particle is given by (5) and hence the acceleration of the particle is given by (6).

$$m = (4/3)\pi\varrho r^3 \tag{5}$$

$$\frac{dv}{dt} = -\frac{9\eta v}{2\varrho r^2} \tag{6}$$

Integrating, we obtain equation (7) from which we can derive the half-life $t_{\frac{1}{2}}$ (equation 8). Substituting the following values, the radius of the protein

$$\ln\left(\frac{v}{v_0}\right) = -\frac{9t\eta}{2\varrho r^2} \tag{7}$$

$$t_{\frac{1}{2}} = \frac{2\varrho r^2}{9\eta} \ln 2 \tag{8}$$

$r = 10$ nm, its specific gravity $\varrho = 1.3$ and $\eta = 0.8$ mPa s (mN s m^{-2}) for the viscosity of water, we obtain $t_{\frac{1}{2}} = 2.5 \times 10^{-11}$ s. This time is remarkably short and becomes even shorter as r decreases. Even if the viscosity was as low as that of the air (0.02 mPa s) $t_{\frac{1}{2}}$ would still be only 1 ns. The crucial conclusion we must come to is that no energy can possibly be stored in the kinetic energy of a protein (McClare 1971).

THE DIFFICULTIES OF STORING ENERGY IN PROTEIN CONFORMATIONS

From these experiments we conclude that ATP energy is transferred to the

myosin where it is stored for some minutes in an available form which may be tapped and converted into external work by the actin. But ATP is stable in physiological solutions ($t_{\frac{1}{2}} \approx 2$ yr; Hulett 1970) so why is it hydrolysed *before* the actin is in position? How is this released, available energy specifically converted into useful work? What traps it and prevents its decay into heat? Such questions are simple to ask but extraordinarily difficult to answer.

The problems we face are basically those of storage of energy and of specificity. Now, energy is stored by displacing a force and ensuring that both force and displacement last for longer than τ, the time needed for a complete cycle. So, we must consider what forces are appropriate for this purpose. However, in a *cyclic* process if molecules have to associate and undergo special changes at one point, then necessarily they have to come apart again later. If we consider the kinds of forces normally used to explain biophysical phenomena, we see that when molecules attract each other they pass from a high free-energy state, in which the force between the molecules is least, to a lower free-energy state in which the force between them is at its maximum. In particular, these forces are *static*; they still exist *after the energy which was stored using them has dissipated.* For example, at a large separation two opposite electric charges are at their greatest potential but the force between them is least but, after the potential (i.e. stored) energy has been lost as heat and an electrostatic bond results, the force between them is greatest. This property is the very opposite of what we want; it means that the separation of the molecules later in the cycle also requires stored energy. This conclusion applies to hydrogen bonds, covalent bonds, London (Van der Waals) forces and (provided that the system is isothermal) to entropic forces as well. Owing to the viscosity of the medium these forces are not conservative; kinetic energy is continually lost at the expense of potential. In both the making and breaking of bonds some stored energy has to be lost. (Incidentally, this problem of an early change in a cycle having to be undone at a later stage is also encountered with the Carnot cycle; it is solved there through the lower temperature for the return stroke. This solution is not available to us; our cycle is isothermal.)

Consider also the effects of Brownian motion. Thermal motions are unspecific and *cannot* be stored; therefore, they are not suitable for making a molecule do something at a specific time. Furthermore, thermal motions are continually disruptive and, although they may be controlled with potential barriers, for example, such barriers have to be erected and removed at specific times in the cycle which means that still more stored energy will be wasted. Yet even with an activation barrier of, say, 12 kJ/mol (which would require almost one third of the available stored energy) 1% of all collisions would still be energetic enough to eject a bond from a potential-well of this magnitude

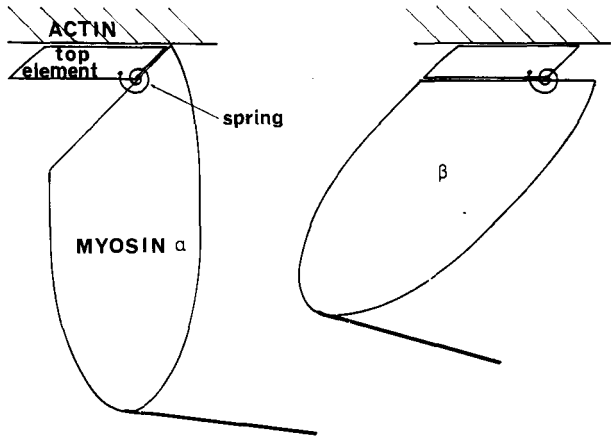

FIG. 1. The 'clothes-peg' model of muscular contraction. In state α the spring at the hinge between the main body of the protein and the top element is twisted so that, when the top is attached to actin, the molecule tilts as it relaxes to state β—thus pulling on the rod at the bottom.

because $\exp(-12/RT) \approx 10^{-2}$; this means that such a bond would last only about 10^{-10} s in water.

For these reasons I concluded (McClare 1971, 1972*a*,*c*, 1974) that, although conformational changes are very important in allostery and in enzyme action, such changes are not suitable for storing the energy released during muscular contraction. I can illustrate these arguments by analysing in detail how one might try to use a conformational change for this purpose. The following model is naive and numerous alternatives will be obvious at every step, but the essential point is the subsequent effect of any particular step, so that it is important to follow any suggestion through to the end of the cycle. Also, simple mechanical forces are used because they are easier to visualize, for it is the static nature of the forces that matters; the argument would be no different if more sophisticated forces were used. In addition, studies of conformational changes at atomic resolution have shown how readily proteins like haemoglobin (Perutz 1972) and lysozyme (Blake *et al.* 1967) can be understood in such simple terms. So, if we postulate that myosin has two states, α and β, of high and of low energy, respectively, we could imagine these as the twisted, α, and relaxed, β, forms of a spring (Fig. 1).

To model the ATP itself is no more difficult; we can imagine it as two charged spheres connected by an inextensible thread (the legitimacy of this model will

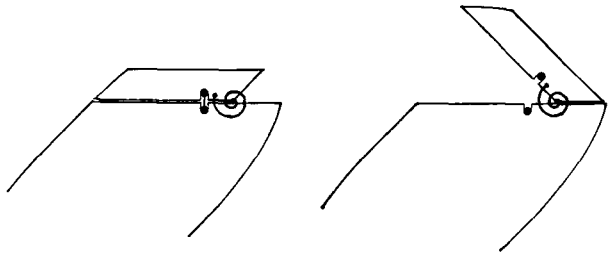

FIG. 2. Energy is stored in the spring by the repulsion between the two charged spheres (●) which represent ATP. The spheres are initially held together by a thread which is cut, an action signifying the hydrolysis. The electrostatic potential is thus used to produce a conformational change which stores energy.

be shown later). Hydrolysis of ATP then corresponds to cutting the thread, but the problems of doing this at exactly the right place and time will not be tackled—even though they are considerable—it will simply be assumed to happen when we want it to. After hydrolysis, the spheres would fly apart and could be used to push the protein into state α (Fig. 2). For the spring to be extended however, the force exerted by the released charges must be greater than that exerted by the spring and yet (we must remember that the system is highly damped—see before), the top-element of the myosin can only move to the position where the spring exactly balances the force between the charges in their new position. Torsional energy could be stored in the spring in this way. (It would amount *at most* to 12.5% of the original electrostatic potential energy, as can easily be shown; but I shall let that pass.) Before this stored energy could be used, however, the charges would have to be released for they hold the protein in state α. The top element would then spring back and, if it was attached to the actin, would cause tilting.

The main cause of inefficiency in this model, so far, is that, immediately after release, the charges experience no opposing force. At first sight, therefore, a better alternative appears to be to have the protein-spring squeezing the 'ATP' before hydrolysis occurs. But then to get the charges into place before hydrolysis, energy would have to be used which could only come from thermal motions. In this case ATP energy could be dispensed with; hence, this alternative is impossible.

Clearly, the charges must not be allowed to escape from the protein too soon but only when the actin has bound, hence we have to put them in a potential-well safe from the disruptive effects of Brownian motion. We could then arrange for the actin to push the charge out of this site (represented in Fig. 3

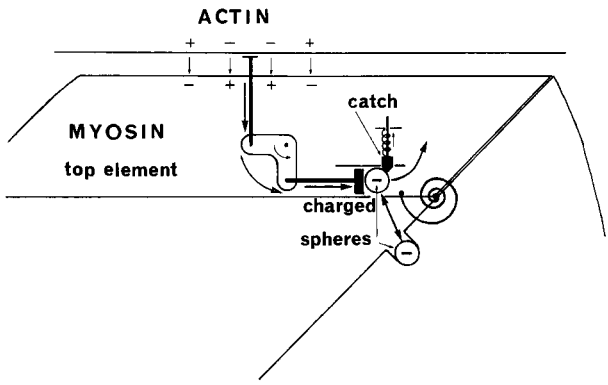

FIG. 3. A mechanism for releasing the charge. The attraction between the actin and myosin causes a conformational change which pushes out the charge which was holding the top element in state α. This process is represented (with inspiration from Heath Robinson) by a rod which transfers the thrust from the actin by a rotating **L**-piece to a piston that pushes the charge past a spring-loaded catch (which is necessary to stop thermal motions shaking the charge out prematurely).

simply by a spring-loaded catch) so enabling the head to tilt. To complete the cycle, however, the actin and myosin have next to be released from the resulting 'rigor' position. This step could be effected by the next ATP as it binds and, by the same mechanism as in Fig. 3, an attractive force on the ATP can be supposed to push back the piston and thus separate the actin and myosin. But here we encounter a considerable difficulty because we could not use the charges themselves (for example, have two positive charges in the binding site to pull the ATP in) because, since there would then be no residual net charge force left after the thread is cut, state α could not be attained. However, if we invoked a different interaction (e.g. H-bonds) to pull the ATP in and separate the actin and myosin, this interaction *would still exist in state* α so that the charges could not be ejected and the cycle would stop at state α.

Thus, the above approach is fundamentally mistaken. Careful analysis shows that if a problem is removed at one step it reappears, like the heads of the Hydra, at another; it is never enough to solve one step in isolation because that solution alters steps later in the cycle. But the central difficulty with this kind of mechanism is the waste inherent in it, for the *only* way to save energy is to ensure that all the forces used are conservative. Yet, when the actin attracts the myosin, the force used is opposed only by viscosity and by the catch-spring (Fig. 3). For the catch to be effective in restraining the charge earlier, the energy used to operate the catch must be wasted; thus, neither

force is conservative. The energy for each step in the above cycle is either wasted completely or is used inefficiently because the conservative force (e.g. that between the charges) is opposed by another which is too weak.

THE RESULT OF ACCEPTING THAT REVERSIBLE PROCESSES MUST BE SLOW

Now, it is just at the above point that the classical treatment of thermodynamics has misled us, for we have assumed that this imbalance of forces is inevitable and (since after all we know that muscles do perform work) we have concluded that there cannot be any fundamental objection to having unbalanced forces. But this, largely implicit, argument assumes without question that no alternative approach is possible. This assumption arises because, if we accept that only infinitely slow processes can be reversible, then it necessarily follows that all real processes must be irreversible and inefficient. At the same time, because a process which is at thermodynamic equilibrium cannot exert an external force ($dG = 0 = Fds$) and therefore cannot cause movement, it appears that real processes cannot be at thermodynamic equilibrium either. So, we are caught in a self-consistent circle which leads to inefficiency at every turn: if a process is balanced by an equal opposing force then it cannot change, but if not so balanced it must lose energy. However, although this conclusion is undoubtedly true for many, if not most, situations, its extension to all possible processes is false for it does not apply to mechanical processes at all. The crucial reason why this belief must be rejected in bioenergetic processes is that the only way to avoid the losses above is to introduce *dynamic* forces—like inertial reaction and electromagnetic induction—to balance the applied force against an inertial, conservative force. And while we equate efficiency with infinite slowness, such forces are inevitably overlooked. How dynamic forces can be introduced will be clear after a discussion of another intermediate form of stored energy which is often postulated: a covalent intermediate.

THE DIFFICULTY OF STORING AVAILABLE ENERGY IN COVALENT INTERMEDIATES

It was Lipmann's great insight that the whole function of metabolism is to make as many ATP molecules as possible from the oxidation of glucose (Lipmann 1941) (equation 9, where n is as large as possible). Glucose and

$$C_6H_{12}O_6 + 6O_2 + n\text{ADP} + n\text{P}_i = n\text{ATP} + 6H_2O + 6CO_2 \qquad (9)$$

oxygen are at a higher energy than water and CO_2 (Fig. 4). What this statement means more precisely is that the sum of the energies of the wave functions

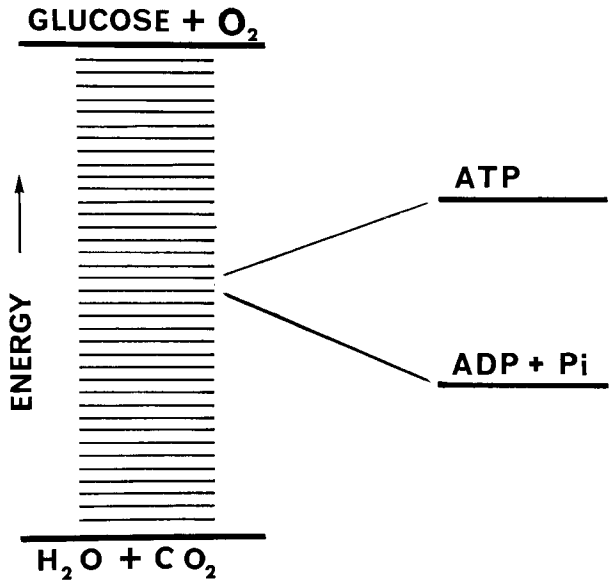

FIG. 4. The overall energetics of metabolism. Energy stored as glucose and oxygen becomes finally stored as ATP. The vertical scale (energy) is expanded on the right to illustrate how one large energy is converted into many small energy gaps.

(which include electrostatic and kinetic energy terms) is greater for glucose and oxygen than for the carbon dioxide and water produced. Somehow, this difference in energy is transferred to many molecules of ATP. In fact, most of these ATP molecules are synthesized during oxidative phosphorylation (which we do not understand either) but some are made by ordinary chemical reactions, for example, that between phosphoenolpyruvate and ATP (equation 10).

$$ADP^{3-} + H^+ + CH_2{:}C(COO^-){\cdot}OPO_3^{2-} \rightarrow CH_3{\cdot}CO{\cdot}COO^- + ATP^{4-} \quad (10)$$

Both phosphoenolpyruvate and ATP are high-energy molecules in that their equilibrium constants for hydrolysis are around 10^6 and the internal energy released as heat on hydrolysis is substantial for each. Thus, in a perfectly legitimate sense (Pauling 1970; McClare 1972*a*), one can talk of the transfer of stored energy between the molecules as the phosphate, or better the monometaphosphate ion (PO_3^-), is exchanged (Fig. 5).

Thus, chemical intermediates certainly allow transfer of energy and so it is a natural extension of this idea to suppose that they are also required in muscular contraction. But no covalent intermediate has yet been found in myosin (Sartorelli *et al.* 1966). If we consider quantum mechanically exactly how

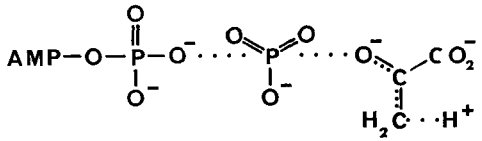

FIG. 5. The transfer of (PO_3^-) between ADP and phosphoenolpyruvate. This process is accompanied by the transfer of stored energy.

energy transfer is effected here it becomes clear just why chemical intermediates are no more appropriate for doing external work in muscle than are conformational changes.

The problem is how to transfer energy without causing hydrolysis at the same time. This question must be carefully separated from that of catalysis; the latter is solved (as is well known) by lowering the activation energy for transfer of PO_3^- while maintaining the barrier against hydrolysis—this explanation is by no means questioned here. The present problem is more fundamental: what is it about the bond which enables a group to be exchanged and, even if there were no activation barrier to exchange, the energy transferred not necessarily decay into heat? The answer is not easy, but it is illuminating.

RESONANCE IN BOND FORMATION

The electronic configuration of a molecule can be looked at in two complementary ways: as a stationary state describable only by probabilities or as a real, fluctuating wave which alters when perturbed experimentally. The latter view was that taken by De Broglie and by Schrödinger; Pauling (1960) also uses it in his classical book. Consider the simplest possible molecule, the hydrogen radical-ion, $H_2^{+\cdot}$. Pauling explains that bonding comes about by *resonance* between the two waves of equal frequency corresponding to the two, unbonded, forms ($H^{\cdot}$ H^+) and (H^+ $H^{\cdot}$) which couple together in two ways like classical oscillating springs: either with the displacements opposed, producing a higher frequency, or together, forming an oscillation of lower frequency (Fig. 6). With the H_2^+ system there results a new (anti-bonding) orbital of higher frequency and energy and also a new (bonding) orbital of lower frequency and energy. This explanation sheds light on three of our problems: (*a*) how a bond is formed from unbonded atoms; (*b*) the nature of the bonded state at thermal equilibrium and (*c*) how a group which is already bonded may be exchanged.

Consider first how a new bond is made. As two unbonded atoms approach

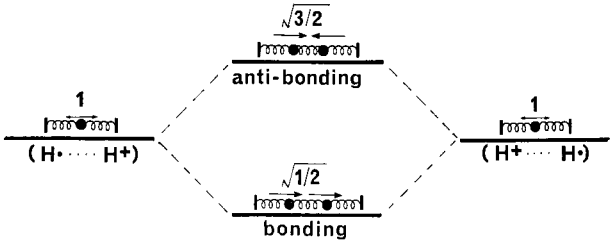

FIG. 6. Quantum mechanical coupling between waves of the same frequency. The two waves couple by resonance rather like classical springs in that a higher frequency (and energy) mode results if coupled out of phase and a lower frequency when coupled in phase. (The numbers give the relative frequencies of the classical systems.) The difference in energy between these bonding and anti-bonding orbitals is zero at infinity and increases as the nuclei approach.

each other (in a vacuum) the force between them is conservative so that the total energy (potential + kinetic) remains constant, but the difference in energy between the bonding and anti-bonding orbitals gets greater as they approach so that the system cannot remain exclusively in either orbital but has to *exchange* between the two as the nuclei approach. Within a certain separation, however, the repulsion between the nuclei dominates because the electron-wave becomes too confined if it remains mostly between the nuclei, and the atoms fly apart again. Hence, only if some of the energy is removed can the molecule stay together. The excess of energy could be removed conservatively as radiation or wastefully by collision with another molecule; the latter is usually the case. Consider the system after it has lost not all but a little of the excessive energy; it would then *vibrate* and, once more, this motion could be described as a series of transitions between bonding and anti-bonding orbitals. Thus, the difference between atoms, or groups, making a bond and coming apart again, and the same system vibrating is conceptually, and energetically, very small. I shall return to this point later. To get into the ground state and attain thermodynamic equilibrium with its surroundings, the system has to lose all the extra energy; again, usually as heat. Finally, in the fully-bonded state, the molecule can be described in terms of the lower orbital alone or, equivalently, as *resonating* in phase between states in which the electron is on one or the other atom.

This last concept is very difficult for the movement of the electron is 'virtual' and is not real in the sense that energy could be taken from the exchange, for the system is already in its lowest energy state, nevertheless these exchanges have real effects: they are the zero-point fluctuations which give rise to London dispersion forces for example (e.g. Bohm 1951); they also are what enables

the energy of PO_3^- to be transferred conservatively. If we consider the actual bond transfer in Fig. 5, we can see that this process is by no means the same as the formation of a completely new bond (when energy would have to be lost again) but is a resonant process (which is difficult to visualize) in which virtual transitions take place between wave-states corresponding to $CH_2{:}C(COO^-){\cdot}O^-$, PO_3^- and ADP^{3-}. Hence, at no time need these transitions couple to thermal motions because they are of the same character as zero-point fluctuations. However, and this point is crucial, the analysis shows that, in general, that part of the energy which is so transferred would be *just as much stored in the bond as it was before transfer*—necessarily so or intermediates like phosphoenolpyruvate (or glucose itself, for that matter) could not store energy. There is no reason why covalent intermediates should not be found on the protein during muscular contraction but their presence would not explain anything because the problem of subsequently making the energy available for external work would remain just as unanswered.

The above analysis also shows that our naive model of ATP—two negatively charged spheres held together by a thread—was perfectly adequate for the conformational-change model of muscle because the Hamiltonian function for a state gives its energy and this function includes both electrostatic and kinetic terms. But, because of the viscous resistance of the medium, we can ignore kinetic energy for it has to be wasted and hence a purely electrostatic model for ATP is all that we need for that approach. On the other hand, this same argument leads naturally to the suggestion I have made elsewhere (McClare 1972*b*,*c*) that the hydrolysis of ATP initially releases stored energy *as a bond vibration.* This proposal solves many of the problems discussed above: first the instant a bond is broken the force produced is balanced by inertial reaction—a *dynamic* force—and, as in classical mechanics, this situation readily produces an oscillation because the forces involved are conservative; secondly, an individual bond is smaller than the surrounding molecules so that a bond-vibration is not subject to viscous drag; thirdly, the energy is necessarily highly specific owing to its inherent frequency so that the transfer and use of this energy need not be controlled by activation-barriers, the shapes of molecules and the like, but is an automatic property of a bond-vibration, provided that an energy level with an identical frequency also exists. Finally, the means of producing a force and converting the energy into external useful work closely resembles the transfer of energy between phosphoenolpyruvate and ADP for, once more, it is a resonant process. The difference, however, is that whereas PO_3^- is transferred between molecules by zero-point fluctuations in the *ground* state, when an excitation is shared between two identical oscillators—like two identical bonds or, better (because the transition-dipole is far larger), two

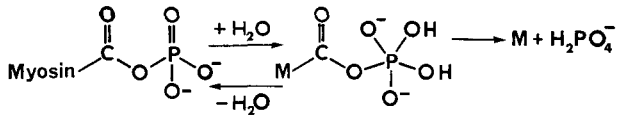

FIG. 7. Possible intermediates in the hydrolysis of ATP by myosin. (The five-substituted phosphorus atom could exist as a trigonal bipyramid.) This intermediate should be hydrolysed far faster than it should decay back to the acyl phosphate.

identical electronic states (McClare 1972*b*,*c*)—all the excitation energy may be taken from the system and then *the force between the molecules disappears* (or rather it decreases to a mere dispersion force). This is the way that the central problem of the other approach, namely, that the force between molecules is greatest after their stored energy has been used, can be solved in a simple way. Furthermore, from this viewpoint the evidence from ^{18}O-exchange experiments suggests that a bond-vibration has already been detected.

THE EVIDENCE FROM ^{18}O-EXCHANGE

Koshland & Levy (1958) showed that when ATP is hydrolysed by myosin in $H_2{}^{18}O$, instead of the one ^{18}O atom expected for terminal P–P fission being found in the P_i released, as many as three were detectable. They also showed that myosin neither catalyses the exchange of ^{18}O into ATP itself nor into ADP and that the direct exchange into P_i, although detectable (Dempsey & Boyer 1961), was insufficient to explain that observed during ATP hydrolysis (Koshland & Levy 1964). This evidence was interpreted, very reasonably, as indicating a covalent high-energy intermediate in the reaction. One possibility, an acyl phosphate, is shown in Fig. 7. The difficulty with such suggestions is that we have every reason to suppose, on purely chemical grounds, that the rate of the final step in Fig. 7 is far greater from left to right; this, after all, is what a high-energy intermediate means. Sartorelli *et al.* (1966) exhaustively searched for an intermediate using many different techniques but failed to detect one. Finally, they proposed (apparently a little reluctantly) the formation of monomeric metaphosphate in the active site.

This suggestion requires that a special environment should have evolved where the ordinary chemical reaction (11) could take place about as rapidly

$$PO_3^- + H_2O \rightleftharpoons H_2PO_4^- \qquad (11)$$

in both directions. There may be reactions in which PO_3^- is transitorily produced (Clark *et al.* 1961; McClare & Wild 1965) but, if so, it is very short-

lived and is certainly a powerful phosphorylating agent which reacts with P_i to form pyro- and meta-phosphates, so that the reaction of PO_3^- with water must normally have a large drop in free energy. Suppose, therefore, that in the protein the tetrahedral $H_2PO_4^-$ ion was bonded to three H-bonding groups so sited that they strain the ion towards the planar PO_3^- configuration and thus stabilize the monometaphosphate ion itself. Then, the free energy of reaction (11) would be far less. But, in this case, the ATP would be hydrolysed with a substantial free energy loss and another loss would be made as the P_i passes into the medium. Moreover, apart from such difficulties, it would remain (as argued above) just as hard to see how such an environment could later be used to produce a force on the actin—which is the object of the whole exercise.

As we saw above, the difference between PO_3^- and H_2O forming a bond and coming apart again, reaction (11), and the (HO)—(PO_3H^-) bond *vibrating* is very small energetically and quantum mechanically. Thus, if the product of hydrolysis is not PO_3^- (which has a high energy *ground*-state) but, as postulated elsewhere, either the phosphate or some other part of the ADP–P_i–Mg complex in an excited vibrational state, then ^{18}O exchange should be possible because only a small activation energy would be necessary to effect it. In other words, because a bond vibration is quantum mechanically equivalent to transitions between bonding and anti-bonding states, the suggestion of Sartorelli *et al.* closely resembles the vibrating bond hypothesis.

Unfortunately, this availability to water and the remarkably long lifetime raise serious problems with this model, too.

THE DIFFICULTIES OF THE VIBRATING BOND THEORY

When I first proposed the vibrating bond theory (McClare 1972*b*,*c*), the lifetime of this state seemed such a problem that a mechanism was devised to allow one reaction to trigger the next by transfer of a phonon (vibrational quantum) so that only the first vibrational state produced need be exposed to the medium, and then for only about 0.1 μs. However, with the demonstration that the high energy state lasts for minutes (Yamada *et al.* 1973), it is clear that, unless this lifetime can be explained without invoking the thin filament, this model is false. If we consider carefully the mechanism of vibrational relaxation, however, even lifetimes of minutes become at least conceivable.

In the first place, the upper limit to the lifetime is set by radiation damping, for an excited state is like an oscillating dipole which radiates by coupling inertially to its electromagnetic field. The rate at which energy, E, is lost

depends on the frequency of the oscillation, ν, and on the strength of the dipole, μ (equation 12), where K is a constant (e.g. Bohm 1961, p. 439).

$$\frac{dE}{dt} = K\nu^4\mu^2 \tag{12}$$

With visible fluorescence lifetimes of 10 ns are often observed. The postulated vibration, however, would have a frequency some 10 times less and, as is usual for infrared transitions, a dipole some 100 times smaller. These factors would raise the radiative lifetime to around 1 s and, if the vibration produced had any smaller transition-dipole, this could well be increased to a minute. Unfortunately, however, vibrations rarely relax by radiation, usually by collision; relaxation times of from 10^{-9} to 10^{+1} s have been observed in gases (e.g. Stretton 1969). Liquids have been studied less but appear to have more rapid relaxations due to the 100-fold increase in collision rate. The theory of relaxation (Schwartz *et al.* 1952) agrees well with experiment and shows that all the vibrational energy, which, as i.r. spectroscopy shows, has a precise value, has to be completely converted into a combination of vibrational, rotational and translational modes in the colliding molecules. Hence, this theory predicts that a bond placed in a site in which neighbouring bonds are far from resonance (preferably of higher energy), rotations and translations are minimized and nearby bonds are *oriented* so as to minimize coupling, then the lifetime of a vibrating state might well approach its radiative limit. In other words, vibrational relaxation in fluids (especially unstructured fluids) where relative orientations can continually change, may well be extremely different, and much faster, than in solids. So, although they certainly must not be ignored, these difficulties may not be fatal to this theory.

In conclusion, the need for conservative forces; the similarity of resonant exchange of (unavailable) stored energy in the ground-state (in PO_3^- exchange) to the resonant exchange of (available) stored energy in the excited state; the remarkable similarity of bond vibration to bond dissociation (thus explaining the ^{18}O exchange)—all appear to converge on the one solution: a vibrationally excited bond is the initial product of ATP hydrolysis.

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Discussion

McGlashan: You began, Dr McClare, by distinguishing between molecules and systems. Of course, an individual molecule has no thermodynamics. When you refer to a cyclic isothermal molecular machine, do you mean a machine in which a single molecule performs some cycles and produces work? It does not seem to be impossible to imagine such a machine. But why drag in the word isothermal? The molecule does not have a temperature.

I begin to wonder why you are worried about bringing in classical thermodynamics at all. After all, if one makes a molecular beam, the molecules are essentially independent and have nothing to do with thermodynamics; they are not in Boltzmann distribution. A beam of molecules travelling along a straight line with a uniform speed can drive a turbine round—that is entirely a mechanical matter. The quantity of work obtained is given by the kinetic energy lost by a molecule to the blades of the turbine multiplied by the number of molecules, which, if the work is about 1 kJ/mol and the velocity of the beam is about the same as the average speed of molecules in thermal equilibrium, is about 10^{24}.

McClare: In a vacuum one molecule by itself would still perform a unit of work even with this system.

McGlashan: But if they behave randomly, the system would produce no work at all; all 10^{24} molecules must be acting cooperatively. Why are you worried about one molecule or, alternatively, why are you worried about classical thermodynamics?

McClare: It seems to me that there is a difficult and fundamental conceptual problem in reconciling the second law of thermodynamics with Brownian motion. As Popper (1957) has emphasized, if we define doing work simply as displacing a force (e.g. lifting a weight), then we also have to say that, since molecular motions lift weights, heat is continually being converted into work in cyclic isothermal processes; so we are left with the paradox that the most fundamental property of heat—molecular motion—appears to break the basic law of thermodynamics. The way out of this paradox is to realize that lifting a weight is not enough; it has to be lifted onto something and *trapped* there, and when you ask how long, this introduces a time-scale.

Gutfreund: The conceptual difficulty of one molecule and temperature is obviated in these models of muscle because the system is bathed in a solvent. We can then apply normal kinetic theory. The argument about one molecule is futile.

McClare: I agree that there are many solvent molecules, but the problem of describing the behaviour of one independent molecule of one type in the presence of millions of another type still remains. The solution is to use time-averages. Professor McGlashan remarked that one molecule cannot be said to have a temperature, but this is only true for an instant; if we average its kinetic energy for a long enough time and divide by twice Boltzmann's constant, we obtain its temperature.

McGlashan: No: we do not. Your 'temperature' is at any rate not the thermodynamic temperature. But I asked the question rather carefully: 'Why are you worried about one molecule or, alternatively, why are you worried about classical thermodynamics?'

McClare: I entirely accept classical thermodynamics at the macroscopic level, but I have shown that (contrary to the view usually accepted) the very same logic requires it to say definite things at the molecular level, too. Then, the approach which has been used up to now cannot work. In all the chemical models put forward so far, ATP energy is effectively converted into heat that is then used in a cyclic isothermal process; this is impossible for the energy has to remain stored even during transduction (McClare 1971, 1974). The conformational-change model and the covalent-intermediate cannot explain where the energy is coming from.

McGlashan: I do not accept that you have shown any such thing. In any case, thermodynamics won't tell you the mechanism, and the mechanism is the real problem.

Gutfreund: The system still has to follow thermodynamic laws: at a given temperature, it has a particular kinetic energy. Professor McGlashan is simply saying that you should forget your criticisms of thermodynamics, which is irrelevant to your problems.

McClare: With respect, I disagree. It is extremely relevant for the reason that muscle is undoubtedly a chemical machine and chemical thermodynamics was evolved for precisely such a problem. Yet what does thermodynamics, as ordinarily expressed, tell us about what a single projection on a muscle can or cannot do? It tells us that we cannot convert heat into work in a cyclic isothermal process. But we can have no idea what this is forbidding until we can distinguish between heat and work at the molecular level.

McGlashan: Thermodynamics tells us nothing about a single projection on the muscle. It does provide some limitations and some relations between observed quantities for the whole system.

McClare: It tells us about relations between time-averages for a single molecule.

McGlashan: In a whole muscle?

McClare: For a single projection.

McGlashan: No, it doesn't. Nobody ever said it did.

McClare: Einstein (1917) did; he predicted stimulated emission on precisely this basis. He realized that a single molecule at equilibrium with a thermal radiation field in a vacuum would not obey Boltzmann's distribution law unless stimulated emission occurred.

McGlashan: But that is precisely the point that Professor Gutfreund has just made: a radiation field is a system.

McClare: Yes, I accept that. Nevertheless, if we had one molecule in an evacuated glass flask inside a black body, we would still be left with the problem of understanding how *one* molecule could obey a distribution normally expressed in *numbers*. Einstein did this by replacing the *number* in each state simply by the average *time* spent in each state. This approach then becomes applicable to molecular machines.

Huxley: Can't you apply this approach to the average behaviour of many cross-bridges, which, after all, is what we measure?

McClare: Yes, we can, but we must distinguish between two kinds of average here: a number-average, which is what we measure, and a time-average, which is more fundamental. As a simple example, we could test the statement 'all conscious people spend one tenth of the time blinking' in two ways: either

by taking a photograph with a rapid exposure of a crowd and counting the number blinking, or by taking a cine-film of one person over an extended period. The crucial difference between these two tests is that the still photograph would tell us the ratio of times alone while the cine-film would also tell us the time between the blinks. The ergodic hypothesis is equivalent to saying that if the crowd is large enough the time between blinks does not matter if we are only interested in the proportion of the total time spent in each state. This is perfectly true but, for molecular machines, we have to know more; we need the time-interval, too.

McGlashan: Be careful about the kind of time average. A muscle is not a system in thermodynamic equilibrium; you are describing a steady state, which is a different kind of time average from the one involved in ergodicity.

McClare: Yes, but we also have to be equally careful about the kind of equilibrium. A system can be at equilibrium relative to one process and yet very far from equilibrium relative to a different process. For example, the heat source and heat sink in a perfect, reversible Carnot cycle are very far from equilibrium relative to direct conduction of heat between them but, with respect to exchanging heat with the heat engine or, by means of the heat engine, with each other, they are at equilibrium. Similarly, a sound-wave is in a state of dynamic mechanical equilibrium and an individual molecule in the gas carrying the wave is in thermal equilibrium with its immediate neighbours, yet, neither the wave, nor that molecule, are at complete equilibrium because eventually the wave will die away. A muscle is not at equilibrium only in this last sense—in just the same sense that a lifted weight on a table can be at thermal equilibrium with the room and still capable of falling to the floor. I suspect that a projection is at thermal equilibrium with its environment always; that the force it produces is at mechanical equilibrium; that the thermally-exchanging energies of ATP and the system are at equilibrium with each other (but not with the mechanical, stored, energies) and that the stored energy becomes converted into external mechanical energy also at mechanical equilibrium. It is possible to make these distinctions simply because each process has a different time-scale.

Taylor: The point you illustrated in Fig. 3 is important: the building of a model which is efficient presents great difficulties. This is irrelevant to arguments about a single molecule and thermodynamics. We respond psychologically in different ways to this problem. Perhaps, one can understand the subtlety of it from the chemistry of the system, and that is where I disagree with you, in thinking that we don't have to put forward another class of mechanism. Any acceptable mechanism must be smart, as Professor Huxley pointed out, to make the process reasonably efficient.

Huxley: I suspect something quite elementary would be sufficient to allow high efficiency—perhaps like my 1957 theory but with the addition of reverse rate constants appropriate for the equilibrium constant of ATP splitting, and binding constants that decrease with the energy in stretching the spring. If we assign the values we want to the rate constants, within the limitations imposed by equilibrium constants, there is no difficulty in getting as close as we want to 100% efficiency. There does not seem to be any difficulty in principle, with the usual qualification that the efficiency is 1.0 minus something of the order of $-v/kd$, where v is the speed, k the rate constant and d the distance over which a cross-bridge works.

On the evidence that Dr Trentham gave us (pp. 69–81), the main part of the energy step comes before the splitting of the bond in ATP. I therefore wonder whether the actual mechanism of the splitting is relevant.

Trentham: From the experimental evidence with the myosin ATPase, the equilibrium constant for the binding of ATP is large and the subsequent equilibrium constants are quite near unity. In terms of the free-energy profile of the process of reaction, the myosin–products complex is in an energy trough.

Huxley: Further, the reaction of bond splitting in ATP is almost at equilibrium while the ATP is bound to the enzyme. The bound ATP is formed quite readily from ADP and P_i in solution, but this ATP does not detach unless the enzyme is destroyed with acid. If we may speak of energy being released in the process ATP $\rightarrow$ ADP + P_i, most is released at stages *before* the bond in ATP is broken, that is, in the stage of binding and in some event in the protein after ATP is bound that is associated with the change of fluorescence.

Cohn: Is the rate of the bond-splitting step neither the rate-determining step nor determined by the equilibrium?

Taylor: Certainly, the bond splitting is not the rate-determining step. However, the second statement still worries us because of the effect of the actin binding on the equilibrium.

Cohn: Is the splitting of ATP into ADP and P_i in the presence of myosin consequently energetically unimportant?

McClare: By no means. In a muscle extracted with glycerol or poisoned with fluorodinitrobenzene, ATP has to be the ultimate energy source; the energy has to come from the enthalpy change on hydrolysis.

Lipmann: Can you define chemically what happens when the ATP binds to myosin, because apparently the energy is displaced into the bond between the ATP and protein?

McGlashan: Is anything known about that bond?

Taylor: No; just as we cannot answer that for any other enzyme unless we know the conformation of the enzyme.

Lipmann: If energy is used in the binding, is then still more energy made available by the splitting?

Huxley: The greatest energy change occurs in a step between binding and splitting.

McGlashan: How do we know that?

Huxley: If the reaction between enzyme, ADP and P_i is quenched with acid, the amount of ATP that comes off is many orders of magnitude greater than could be obtained from the equilibrium mixture of these small molecules in solution.

Whatley: Does the myosin provide the energy for the dehydration? If so, myosin clearly has enough energy for the synthesis of ATP in this particular case, except that we do not know how to release it except by swamping it with acid.

Trentham: Boyer *et al.* (1975) have seen the same phenomenon with the mitochondrial ATPase.

Cohn: They also have direct evidence from using ^{32}P and following the formation of [^{32}P]ATP on the enzyme (E·[^{32}P]ATP) and also from the ^{18}O reaction, where the phosphate goes back and forth many times on the enzyme, before reacting with ADP.

Roseman: Is it possible that in the ADP–P_i–enzyme complex, the compounds are represented neither by ADP + P_i nor by ATP, but rather by a resonance state? Acid will then liberate ATP, ADP and P_i, but the enzyme holds the reactants and products together in a transition state.

Huxley: Would that account for the results of the ^{18}O exchange experiments which have also been explained on the basis that the bond is repeatedly made and broken? The bonding is the average of two states, broken and made, rather than being intermediary. While broken, the bond may rotate and incorporate ^{18}O.

Roseman: In a resonating state, the system is limited by the two extreme situations. I'm suggesting intermediate states, such that on addition of $H_2{}^{18}O$, the oxygen atoms would exchange.

Cohn: No; it has been shown that the ^{18}O exchange can be accounted for by reversal of the reaction of enzyme-bound reactants (Boyer *et al.* 1974).

Roseman: Won't $H_2{}^{18}O$ exchange with the phosphate on the protein?

Cohn: Only by the mechanism of forming a bond and breaking it. This happens not only in the energy-coupled reaction, but also in the hydrolysis of inorganic pyrophosphatase (Cohn 1958; Degani & Boyer 1975), where there is no energy coupling. Yeast inorganic pyrophosphatase behaves just like the ATPase in oxidative phosphorylation and myosin ATPase in this respect.

Huxley: This is in keeping with the idea that the splitting step in myosin happens after the interesting energetic steps.

McClare: Sartorelli *et al.* (1966) found no evidence for covalent intermediate in myosin and instead were forced to propose that water is detaching from and attaching to a monometaphosphate ion. This proposal, from a quantum mechanical view, is almost the equivalent of saying that the phosphate produced by hydrolysis is in a high-energy vibrating state (see p. 313). This ^{18}O exchange evidence, and also what Dr Roseman has just said, provides strong support for my hypothesis.

An analogous situation is found with the photooxidation of rubrene. Rubrene absorbs light, forming a singlet excited state which normally fluoresces, but oxygen quenches this fluorescence by reacting with the excited rubrene. The resultant rubrene oxide has only a slightly lower energy than the excited rubrene, which can be regenerated with heat whereupon the excited state fluoresces. If, by analogy, we were to call the rubrene oxide ATP and the rubrene + O_2 in the *ground*-state ADP + P_i, the hydrolysis that releases the energy would be this small thermal step, the R–O_2 to R* + O_2 step, which has a small equilibrium constant; a high-energy compound initially produces a high-energy *state*. Afterwards, the energy in that molecular state is used to do work. So, the fact that the free-energy change in the initial hydrolysis of ATP is small is precisely what we want.

Other results, too, are consistent with this analysis. Yamada *et al.* (1973) showed that although the chemical reaction is rapid, the heat released when ATP is hydrolysed only appears after a matter of minutes. This evidence could only mean that the hydrolysis has a small equilibrium constant and the energy is used (or wasted, in this case) later; it is exactly what we want.

Hastings: In the rubrene system, the emission of light results not from decay of the excited state of rubrene but from the excited state of oxygen.

McClare: I accept that the details are also important and that I may have got them wrong. But this error would make no difference to the argument about the energetics, which is all I used the analogy for.

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Muscle as a thermodynamic machine

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Abstract Three main problems in the energetics of muscular contraction confront us at the present time:–

(1) *Thermodynamics.* This is a labyrinthine subject in the sense that there are often several equally correct ways (that may appear different) of arriving at the same result. The recent advances in the kinetics of the reactions of muscle proteins have increased still further the need for understanding among muscle physiologists – and other biologists – of those parts of thermodynamics that concern them directly, notably those relating work and chemical change.

(2) *Energy balance.* Over the whole cycle of contraction, relaxation and oxidative recovery, the observed energy output (heat + work) can be accounted for by the chemical processes currently believed to operate in the cycle. In contrast, during contraction and relaxation alone, the energy output is greater than can be accounted for by the extent of known chemical reactions combined with their heats of reaction (measured calorimetrically). The physical and chemical methods cross-check one another – hence the usefulness of the method.

The details of this discrepancy may vary from species to species but it is seen in all the experiments known at this time. The general problem will be resolved only when we know more about the chemical reactions that have occurred and reliable measurements have been made of their heats of reaction *in vitro*.

(3) *Work balance.* Work and heat differ thermodynamically and, since the production of *work* is a primary function of muscles, the question naturally arises whether the observed splitting of ATP (combined with estimates of the change in free energy of this reaction) is sufficient to account for the work observed. On present information, which is imperfect in several respects, the chemical change *is* sufficient to account for the work. Estimates of the efficiency of transduction vary in different laboratories from 45 to 66%.

The contractile filaments of muscle that Professor Huxley discussed form a machine made of protein in which a particular chemical reaction, the hydrolysis of ATP, progresses in such a way that it leads to the production of mechanical work. This provides the most dramatic instance of energy transformation.

Since we are concerned with chemical change, heat and work, we are brought face to face with the problems of thermodynamics. From the formal thermodynamic point of view, muscle exactly corresponds to a galvanic cell connected to an electric motor, in which similarly a chemical process occurs in such a way that work is performed.

Now, although muscles are deplorably complicated, they (along with many other biological systems) do at least possess several features that simplify the application of thermodynamics. They are for all practical purposes isothermal systems; the temperature gradients that inevitably arise in them are small and obviously incidental to the way in which the muscle functions. Also, since they form a condensed system in which the chemical reactions proceed in solution, volume changes are negligibly small, about one part in 10^5, so the pressure–volume work done against the atmosphere is always negligible. The difference between changes of energy and of enthalpy is thus also negligible and the difference between Helmholtz' and Gibbs' free energy can likewise be disregarded. By suitable experimental design, we can arrange that the protein contractile machinery and its substrates function either as closed systems (i.e. exchanging energy only with their surroundings) or as open ones (which exchange matter as well as energy with their surroundings). I shall not give a full account of all the relevant thermodynamics (see Wilkie 1960, 1967, 1970, 1974); for our present purposes it will suffice to give two simple equations, the first (1) derived from the First Law;

$$h + w = -\Delta U \approx -\Delta H \tag{1}$$

h is the heat produced, w is the work produced, ΔH is the change in enthalpy and ΔU is the change in energy.

The second equation (2) incorporates the Second Law and tells us that to each specified change there corresponds a maximum value of the work that can be produced (w_{max}); ΔS is the change in entropy resulting from the process,

$$w_{max} = -(\Delta U - T\Delta S) = -\Delta A \approx -\Delta G \tag{2}$$

T is the absolute temperature, ΔA is the change in Helmholtz' free energy and ΔG is the change in Gibbs' free energy. It will be appreciated that in a chemical reaction, the products usually differ both in energy U and in entropy S from the reactants. Equations (1) and (2) apply to a closed but not isolated system in isothermal conditions, in which most experiments on muscle have been conducted. In what follows I shall use the functions ΔH and ΔG because they are probably more familiar and marginally more accurate than ΔU and ΔA.

ENERGY OUTPUT AND CHEMICAL CHANGE: THE METHOD OF ENERGY BALANCE

If only one chemical reaction were proceeding in the muscle, we could write equation (1) in the form (3) where ΔH_m is the molar enthalpy change of

$$(h + w) = -\Delta H = -\Delta\xi\Delta H_m \tag{3}$$

the reaction (in J/mol) and $\Delta\xi$ (in mol) is the extent of reaction during the time interval in question. All the four terms of this equation are determinable experimentally: h with sensitive thermopiles, w with suitable mechanical levers and transducers, ΔH_m by independent calorimetric investigations and $\Delta\xi$ by chemical analysis of the contracting muscle. The determination of $\Delta\xi$ presents the problem that since chemical analysis is a destructive process one cannot analyse the self-same muscle both before and after it has contracted. It is, therefore, necessary to conduct such experiments in duplicate with one muscle as an unstimulated control while the other muscle is stimulated and thus caused to contract in whatever way is under investigation. Both muscles are otherwise treated identically and are rapidly frozen within about 60 ms in a special 'hammer' apparatus (Kretzschmar & Wilkie 1969) in which the muscles are flattened between metal surfaces previously chilled to −196 °C. The muscles can subsequently be extracted with perchloric acid and analysed for substrates of interest.

If all the terms in equation (3) are known, what is the point of the exercise? The answer is that the physical measurements of w and h cross-check the calorimetric and chemical determinations. If equation (3) is found experimentally not to be obeyed (and this is what has been found in all the experiments conducted until now) then we know that our knowledge of the chemical processes must be incomplete, a piece of information that cannot be obtained in any other way. Each additional reaction will contribute its own term similar to the right-hand side of equation (3).

Experimental results

Some years ago the situation appeared to be relatively simple, as shown in Fig. 1 for muscles poisoned with iodoacetate and nitrogen. Biochemical evidence abounds indicating that the contractile system of such muscles forms a closed system in which the only reaction to be expected is the hydrolysis of phosphocreatine. The experiments were conducted on a slow time scale: each pattern of contraction included the phase of relaxation and usually a fair amount of time elapsed between contractions. Since the 'hammer' apparatus

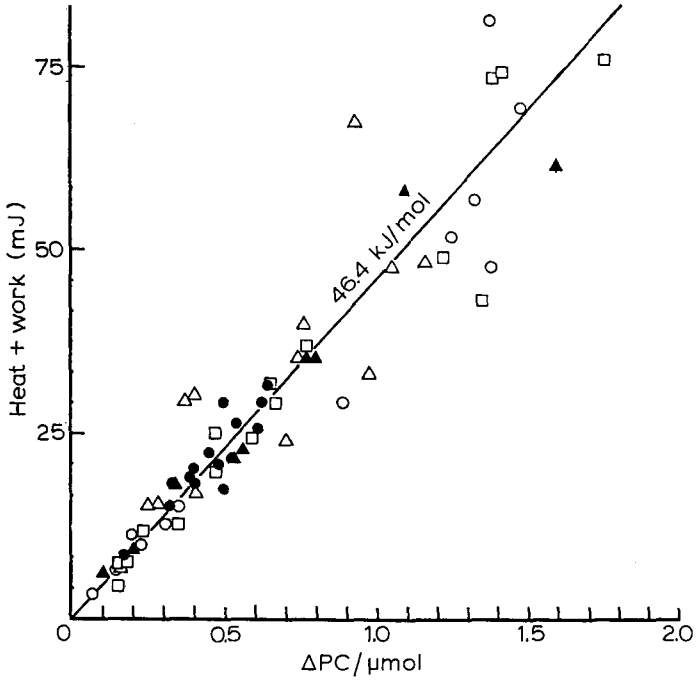

FIG. 1. The relation between heat, work and splitting of phosphocreatine (PC) in various types of contraction including relaxation and an additional delay of about 45 s; muscles at 0 °C treated with iodoacetic acid and nitrogen. Ordinate: (heat produced + work produced) by muscle, equal to the negative change in enthalpy of muscle, in mJ. Abscissa; amount of phosphocreatine split (ΔPC) in μmol: ○, from 4 to 107 isometric twitches, 12 experiments; ●, 30 isometric twitches, 15 experiments; □, isometric tetani lasting from 7 to 111 s, 16 experiments; ▲, from 8 to 78 isotonic twitches, with performance of positive mechanical work, 9 experiments; △, from 2 to 8 tetani, duration 7 s, with slow isotonic stretch and negative work, 13 experiments. The line has been drawn with a slope of 46.4 kJ/mol (as explained in the text). (From Wilkie 1968.)

had not yet been invented there was also an interval of some 40 s between the end of the last relaxation and the moment of freezing.

On this slow time scale, it is evident that there is fair proportionality between the output of energy (i.e. $h + w$) and the breakdown of phosphocreatine. The slope of the line indicates that one is obtaining some 46 kJ/mol of phosphocreatine split. At the time (Wilkie 1968), this was close to the accepted calorimetric value for the molar enthalpy change of phosphocreatine splitting so it appeared that equation (3) was, under these circumstances, being obeyed and as though only one reaction was occurring in the muscle.

Since that time, various discrepancies have appeared which are still not

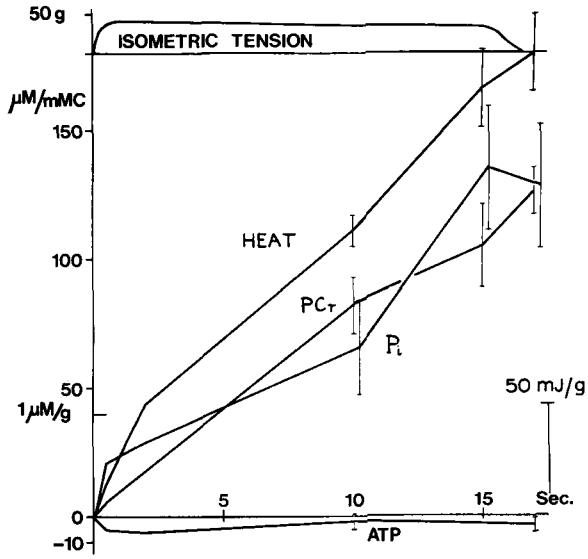

FIG. 2. The relation between heat produced and chemical change during a simple isometric tetanus of 15 s duration at 0 °C; normal muscles in oxygen. Breakdown of phosphocreatine (PCr) and ATP, and liberation of inorganic phosphate are shown as upward deflections. Heat production has been scaled at 46.4 kJ/mol, as in Fig. 1. The upper trace shows a record of tension development and relaxation. (From Gilbert *et al.* 1971).

resolved. In the first place, careful calorimetric determinations (Woledge 1972) indicate that one can expect only 34 kJ/mol for the hydrolysis of phosphocreatine and for the subsequent reactions with the buffers thought to be present in muscle. Secondly, technical improvements of various kinds have made it possible to examine energy balance in contracting muscle on a fast time scale and to follow the output of energy ($h + w$) and the chemical changes from instant to instant during a single maintained contraction. From Fig. 2 it is evident that early in contraction a large amount of heat appears that cannot be accounted for by concurrent splitting of phosphocreatine. The chemical and physical measurements have been matched with the old value of 46 kJ/mol rather than the new one of 34 kJ/mol. Adoption of the latter value causes the discrepancy to become still greater. The obvious inference is that in the early stages of contraction there must be an unidentified exothermic process which we have reason to think may be reversed during a period lasting several minutes after relaxation has ended, as shown in Fig. 3. We have found this result consistently in our experiments on English frogs (*Rana temporaria*), but, to confuse the issue, Homsher *et al.* (1975), who have made similar experiments

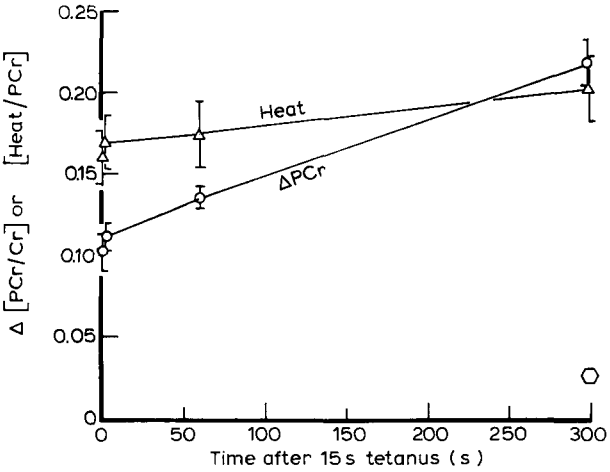

FIG. 3. Postcontractile splitting of phosphocreatine (PCr). The experiment has been preceded by a 15 s tetanus similar to that in Fig. 2. The muscles, at 0 °C, were treated with iodoacetic acid and nitrogen to prevent recovery resulting from glycolysis or oxidative phosphorylation. As in Fig. 2, heat and ΔPCr have been scaled at 46.4 kJ/mol. The hexagon indicates that even after 300 s extremely little fructose 1,6-diphosphate has been formed (Kretzschmar & Wilkie, unpublished results).

on the American frog (*R. pipiens*), find no obvious energy gap early in contractions such as is shown in Fig. 2 though they do obtain 46 kJ/mol of phosphocreatine breakdown. So, their results remain inconsistent with calorimetric determinations. With *R. temporaria*, they obtain results virtually identical with our own. Clearly, this situation demands further experimental investigation.

Work balance

From the thermodynamic point of view, work and heat are very different types of energy. One inevitable question about the contractile machine is, how efficiently does it transduce chemical into mechanical energy? Referring to equation (2) we can compare the work observed experimentally (w) with the maximum that might theoretically be expected (w_{max}), and derive equation (4) (analogous to equation 3); $dG/d\xi$ is often designated by ΔG or ΔG_m (Guggenheim 1967), so care must be taken to avoid confusion. Integration is

$$w \leqslant w_{max} = -\Delta G = \int_{\xi_1}^{\xi_2} \frac{dG}{d\xi} \cdot d\xi \qquad (4)$$

necessary because $dG/d\xi$ alters as the reaction proceeds. Unfortunately, $dG/d\xi$

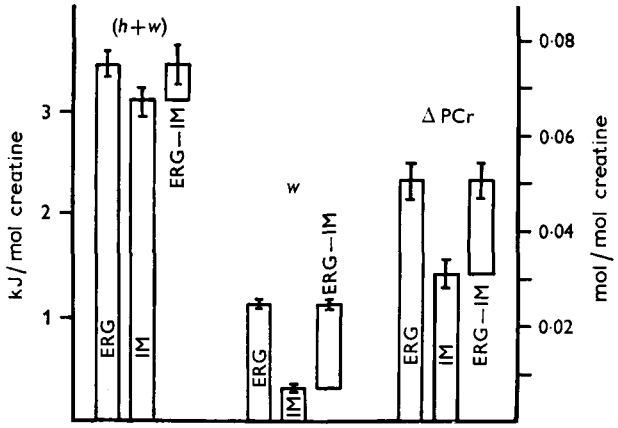

FIG. 4. Heat *(h)*, work *(w)* and breakdown of phosphocreatine (ΔPCr) during working and isometric contractions: ERG signifies a working contraction, IM an isometric contraction. The bars at the top of each column represent ± 1 S.E. So that $(h + w)$ and w on the one hand and ΔPCr on the other may be plotted on a single graph, some constant relating the two must be assumed. Here the constant is taken as 46 kJ/mol. The ordinate is labelled in units of both kJ/mol creatine and mol/mol creatine, all quantities being normalized by total creatine content. (From Curtin *et al.* 1974).

cannot be measured directly at present; it can only be calculated and for this purpose we have used the results of Alberty (1972).

In the experimental approach illustrated in Fig. 4 we compared two brief contractions, in one of which we held the muscle at constant length so that its performance of work was minimized whereas in the other we allowed the muscle to shorten at the speed at which it generates maximal mechanical power. The duration of the non-working contraction (IM) was almost double that of the working (ERG) contraction. We did this to make the total energy output similar in both cases: thus, in one contraction almost all the energy came out as heat whereas in the other about 35% came out as work. The effect on phosphocreatine breakdown is dramatic: it is almost doubled in the working contraction and so the rate of breakdown is practically quadrupled. In optimal conditions we obtained about 26 mJ of work/g muscle and the corresponding (calculated) value of ΔG was about 60 mJ/g muscle. Although heat may be produced by an unknown source, we have no reason to suppose that mechanical work comes elsewise than from concurrent chemical change. On the face of it, the efficiency of transduction appears to be only about 50%. Experiments on this topic (and with *R. pipiens*) were reported several years ago by Kushmerick & Davies (1969) who found higher values of efficiency than we have done (up to 0.66). In their experiments, as in ours, there was always sufficient chemical

change to account for work produced. Note that Fig. 4 provides further confirmation for the result shown in Fig. 2. Phosphocreatine splitting suffices to account for the *work* observed, but not for the *total energy* observed.

THERMODYNAMIC PROBLEMS

Thermodynamics is a labyrinthine subject in the sense that there are often several equally correct approaches (that may appear different) to the same result. As we have seen throughout this conference, biologists are increasingly forced to consider quantitatively the properties of systems in which chemical change is coupled to the output of mechanical, electrical or osmotic work and the emission or absorption of light. Our problem, then, is to find the formulation of thermodynamics that best suits these purposes. Widespread confusion exists about standard and actual free-energy changes in chemical reactions and it may well be that, for reactions in the dilute solutions usually encountered in biology, standards other than the conventional 1 mol/kg might be appropriate. We may even be able to dispense with the idea of such standards altogether, by making more use of equation (5) where K is the equilibrium constant

$$dG/d\xi = RT\ln(Q/K) \tag{5}$$

and Q is a quotient of exactly the same form but relating *actual* concentrations rather than equilibrium ones. It avoids many of the difficulties inherent in other equivalent forms of the van't Hoff equation.

We are faced with a pressing need for more accurate measurements of free-energy changes in systems of biological interest. Most of the calculated values that biologists use must of necessity neglect activity coefficients and various other complexities. Reactions such as the hydrolysis of ATP and of phosphocreatine are much more complicated than simple equilibria because both substrates and products have several binding sites for hydrogen ions and in the case of ATP for magnesium and perhaps sodium ions as well; one consequence is that the main reaction is accompanied by side reactions with fractional amounts of hydrogen or magnesium ions that appreciably alter the free-energy changes (see e.g. Alberty 1972).

On the positive side, the demonstration that both the sodium pump and the calcium pump can be reversed in direction with consequent synthesis rather than hydrolysis of ATP suggests that we may be able to set up a thermodynamically reversible system in which direct measurements of the activities of ions with appropriate selective electrodes could give us direct measurements of the free-energy change of ATP splitting. This would put many calculations, such as those presented in this paper, on a much firmer footing.

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Discussion

Keynes: To eliminate one possible candidate for the source of the extra heat (Fig. 2), can we neglect the heat generated by the electrical conduction process compared with the mechanical heat?

Wilkie: Yes; it is much less than 1%, to judge from experiments on nerve. But the Joule heating by the stimuli presents a greater embarrassment; it certainly is not negligible, but we allow for it, albeit not extremely accurately.

Weis-Fogh: We have studied the reversible stretching of elastin calorimetrically (Weis-Fogh & Andersen 1970). Originally, we imagined that, in such a system, the heat output would have equalled the change in free energy. However, this is not at all the case when water is the swelling agent or diluent. The reversible heat change is then several times larger than the change in free energy. Apparently, when elastin is stretched, the hydrophobic interactions

structure the water. Could such interactions underlie the increased heat output you observe (Fig. 2)?

Huxley: A. V. Hill measured heat changes during changes in a tension stimulated muscle. Professor Wilkie, did you allow for thermoelastic heat?

Wilkie: Yes.

Weis-Fogh: But one cannot do that unless one knows much more about the system, because the free energy is inversely related to heat in elastin (J. M. Gosline, unpublished results). For instance, at a stretch of 30%, the ratio of free energy to heat is 3, but at a stretch of 5%, the ratio has risen to 90.

Wilkie: That means that for a certain input of work, one can get up to nine times as much heat out. But that is an elastic system: for a small amount of work, a tremendous amount of heat can be generated.

Glynn: The discrepancy at the beginning is associated with the period when the slack was being taken up. Could you stretch the muscle initially, so that tension development by the contractile elements was really isometric? If so, you might find that the discrepancy vanished.

Wilkie: You are suggesting a terribly difficult experiment! As A. V. Hill emphasized, the application of a stretch, the putting in of a considerable amount of work, raises the problem of whether the work is dissipated uniformly through the muscle. Further, in order to estimate the heat produced by the muscle itself one must subtract one large quantity (the work put in) from another (the total heat production).

Keynes: What about possible energies involved in calcium release?

Wilkie: At the moment Dr Woledge is investigating calorimetrically whether such large heat changes are associated with the reaction between calcium and troponin. Conformational changes in proteins can generate large amounts of heat, even when the free energy change is small. This is another possibility to account for the heat that we observe.

Woledge: According to K. Yamada (unpublished results, 1973), the binding of calcium to troponin is only moderately exothermic. The reaction provides insufficient heat to account for the unexplained heat produced by muscles. By far the best candidate for an explanation seems to be some change in myosin, which is relatively plentiful and is known to undergo transitions with large enthalpy changes.

Hess: How well is the extent of the reaction determined? Do you use freezing or quenching techniques and do you extrapolate to zero time?

Wilkie: In designing this hammer apparatus, we aimed at freezing the whole muscle in less than 100 ms, otherwise the equipment would have had unsatisfactory time resolution. Since then, we have found out that the centre of the muscle is frozen within 60 ms.

The energy we cannot account for does not depend on uncertainties about freezing time associated with different kinds of contraction (Fig. 4). Imagine a contraction lasting a second. Our total freezing time is less than 10% of that, so the *uncertainty* in freezing time is negligible in comparison with other sources of variability.

McGlashan: The analysis rather than the freezing of the reaction presents the difficulties?

Wilkie: No; the main trouble seems to be the variation between the muscles in frogs. Even after identical treatment of paired unstimulated muscles, we still find big standard deviations in these measurements.

Weis-Fogh: Can you explain the strange difference between American and British frogs?

Wilkie: No. We are planning to get some proper American *R. pipiens* to repeat the experiments.

McGlashan: The amount of work in the isometric contraction in Fig. 4 surprised me. What is that work, and how do you measure it?

Wilkie: In that experiment, to our great shame, we found that our recording apparatus had not been as stiff as it ought to have been. So, about 10% of the total energy was in the form of work; a small fraction (about 3%) is inevitable, but the rest was due to our foolishness in not checking our apparatus properly. In later experiments, we reduced this fraction so that the work done in the isometric case was only about 3% of the total. This work is almost all accounted for in stretching connections and stretching the tendon of the muscle.

Keynes: Your technique gave appreciably lower values of P_i than that of anybody else, a fact which suggests that it is doing well at preventing breakdown.

Wilkie: Yes; we also found high values for phosphocreatine in resting muscles.

Roseman: Besides the glycolytic intermediates from glycogen, did you look at ADP and AMP? Is enough ADP present to generate by the myokinase reaction, ATP and phosphocreatine, and in doing so, produce AMP?

Wilkie: The increase in ATP early in the tetanus (Fig. 2) surprised us. However, evidence has accumulated, persuading us that we had been correct. It seems that early in a contraction ADP is shed from the cross-bridges. In the resting state, they are charged with ADP and P_i, that is, they are primed to perform work. They then shed ADP before they take on a new charge of ATP. The rise in the ATP content is probably due to the rapid rephosphorylation of the small amount of ADP that has been shed.

Roseman: But what is the origin of this mysterious burst of energy? Analysis should reveal any shift from ADP to AMP by known reactions.

Woledge: I have found no change in the amounts of ADP, AMP, IMP or phosphoenolpyruvate which could explain it (Curtin & Woledge 1975).

Lipmann: At the start of the reaction, is only phosphocreatine present or is some split?

Wilkie: In estimates on resting muscles, the ratio of phosphocreatine to total creatine—phosphocreatine plus free creatine—sometimes rises as high as 0.9. So some 10% may be split, either naturally, or as an artifact in our procedure for freezing and extracting the muscles.

Lipmann: I wonder if your equation (5) may be rewritten:

$$\frac{dG}{d\xi} = \Delta G^{\varnothing}_{obs} - RT\ln\left(\frac{[ATP]}{[ADP][P_i]}\right)$$

since the concentration of ATP seems not to change and only the ratio of creatine to phosphocreatine does.

Wilkie: Yes; however I jumped from talking about phosphocreatine breakdown, which we measure, to working out the free-energy change in terms of ATP, ADP and P_i. That is justifiable since abundant evidence has gathered that ATP, ADP, phosphocreatine and creatine are in equilibrium and that, therefore, the free-energy change must be the same for phosphocreatine splitting as for ATP splitting. We measure phosphocreatine breakdown and, therefore, the increase in P_i; we know the initial concentration of ATP, so we can work out from measurements of phosphocreatine breakdown how $\ln([ATP]/[ADP][P_i])$ changes.

Lipmann: Since the breakdown of phosphocreatine renders the medium more alkaline, this change in pH might effect the equilibrium between ATP and phosphocreatine, even to the extent of increasing the concentration of ATP.

Wilkie: No, because at the time we observe the increase in ATP concentration, little phosphocreatine has been broken down.

Glynn: Why is the muscle so much more efficient than the 25% or so that one estimates with a bicycle ergometer? Is the energy transfer between the muscle and the fly-wheel inefficient?

Wilkie: No, but we are looking at the front end of the muscle machine. It's a good question. How much inefficiency arises in transforming the free energy of the splitting of ATP into work and how much arises in rebuilding the ATP that has been broken down, from oxidation and hydrolysis of glycogen and the various other recovery processes? The difference between the initial efficiency and the total efficiency shows that the purely chemical processes involved in rebuilding the ATP account for a quantity of inefficiency almost equal to that arising in contraction itself (Wilkie 1960).

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Mechanical and biochemical cycles in muscle contraction

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Abstract The current biochemical approach to understanding the mechanism by which chemical energy is converted into mechanical energy in muscle is by investigating the kinetics of the reaction of ATP with the isolated contractile proteins in solution with stopped and quenched flow techniques. The results are interpreted in terms of a cycle of hydrolysis of ATP.

The current mechanical approach lies through the kinetics of force production or length change in preparations with the contractile proteins intact, by following step changes in length or tension. The results are usually interpreted in terms of mechanical cycles of activity of cross-bridges, in which a cross-bridge progresses through a series of states with different mechanical properties.

If the biochemist's and the mechanist's cycles are one and the same, a problem arises over the correspondence between states in the two cycles. One approach to answering this question is through the determination of the effect of pH and of such substances as ATP, ADP and inorganic orthophosphate on the mechanical response. We hope that these will act at known points in the biochemist's cycle and their influence upon the mechanical cycle should enable their location here to be ascertained. The paper deals with the effects of phosphate, ATP and magnesium ions on the mechanical response of insect flight muscle.

The length of a muscle changes by the relative motion of two types of interdigitating filament, the thick or A filaments consisting mostly of the protein myosin and the thin or I filaments consisting of the protein actin (plus other proteins—tropomyosin and troponins—which appear to regulate contraction). The most widely accepted theory for the mechanism whereby the two types of filament are caused to move relative to one another suggests that side-pieces or cross-bridges on the A filament provide a mechanical link between the filaments and that these cross-bridges go through an attach–pull–detach–recover cycle of activity, as discussed by A. F. Huxley (pp. 271–279).

The cross-bridges contain the part of the myosin molecule responsible for the ATPase activity of the contractile proteins. Recent work, with rapid

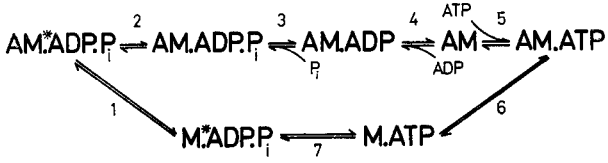

FIG. 1. Possible kinetic scheme for the hydrolysis of ATP by actomyosin (based upon the work of Taylor and of Trentham).

reaction techniques (in particular by Dr E. W. Taylor and Dr Trentham), has identified the individual steps in the hydrolysis of ATP by myosin in solution, providing values for the rate constants of these reactions, and has given an indication of what actin does in the activation of the hydrolysis. The rate constants of the individual steps are such that, in the hydrolysis of the ATP by myosin in the presence of the actin, the actin and myosin associate and dissociate for every ATP hydrolysed, as illustrated in Fig. 1 which indicates a possible sequence of reactions in this hydrolysis. This scheme will certainly need modification as more steps are discovered (with states intermediate to those indicated), but it seems likely that this general scheme will be maintained. In a general review (White & Thorson 1973) the experimental basis for the mechanical and biochemical kinetic schemes is discussed.

An obvious hypothesis is that the mechanical cycle of cross-bridge activity is one and the same as the biochemical cycle of hydrolysis of ATP by actin and myosin. If so, several questions immediately arise.

(1) What is the mapping between the two cycles?

(2) What constraints are placed upon the interactions by the structural relationships of the intact muscle, by comparison with the interactions in free solution?

(3) In particular, how do the rate constants of the individual steps vary with cross-bridge distortion?

(4) What is the tension generated by each attached state and how does this vary with cross-bridge distortion?

Answers to these questions must obviously be found by experimental work on intact muscle, since solution chemistry cannot hope to introduce the steric constraints and the mechanical inputs required. Techniques must obviously be developed for identifying individual 'states' in the intact muscle, and the time course of population changes of these states must be followed after the application of suitable perturbations to the system, such as rapid length changes. Numerous techniques are being tried, including X-ray diffraction, fluorescence

spectroscopy, absorption spectroscopy, electron spin resonance and nuclear magnetic resonance. As these techniques are still being developed, we are not yet able to answer the above questions. (Reports on the progress can be found in *Cold Spring Harbor Symp. Quant. Biol. 37*, 1972.)

A simpler, but less specific method is the measurement of tension in the muscle. The tension developed will probably be contributed to by cross-bridges in several different attached states, and the relative contribution from each state will presumably differ. Thus, a measure of tension will be a measure of the integrated contribution from the particular population of cross-bridges in each of several chemical states. The problem is complicated further by the fact that even for a single 'state', the contribution to tension will depend on the degree of mechanical distortion of that state due to the position of the actin binding site relative to that cross-bridge. This can, perhaps, be understood better by consideration of what happens to a single attached state of a cross-bridge when the thick and thin filaments move relative to one another; the cross-bridge will possibly still remain in the same chemical state but will have been distorted by the movement, and will now exert a different tension. Also, the rate constants for the transitions from that to other states will probably have been modified by the distortion.

Despite the complexity in interpreting the measurements of tension, it is to be hoped that some sense can be made in interpreting the data in which tension measurements have been followed as a function of time after the application of a mechanical perturbation to the muscle. Huxley & Simmons (1971, 1972) have developed a most appealing theory for the initial transients observed after rapid changes of length applied to isometrically-contracting intact frog muscle fibres (see Huxley, pp 271–279).

One way in which the individual steps can be identified in the schemes devised to explain mechanical data is by varying the concentrations of the substrates in the biochemical cycle and determining the effect of such changes on the mechanical kinetics. Several second-order processes in the biochemical cycle involve the association of ATP, ADP, inorganic orthophosphate (P_i) and H^+. Some of these processes are back-reactions in terms of the normal progress around the cycle, but the ATP association is a forward reaction. Also, as the effects of a number of other factors (such as ionic strength and temperature) on the biochemical kinetics are known, their effect on the mechanical kinetics may be informative.

Several problems hinder the design of experiments to measure the mechanics of muscle in the presence of different chemical environments:—

(1) It is not possible to use normal intact live muscle fibres, because these possess numerous control processes which buffer most ions of interest,

especially nucleotides. Several ways of getting round this problem have been tried, for example, 'skinning' the fibre of its outer membrane or sarcolemma with forceps, or extracting the contractile proteins intact by chemical procedures (of which the earliest is glycerol-extraction). Many other methods have been attempted, including the use of detergents to attack membrane systems and ions such as EDTA to change the permeability of the outer membrane. We have mainly used the glycerol-extraction procedure.

(2) Given a suitable preparation of the contractile proteins, these need to be bathed in a solution which remains uniform in its constitution throughout the body of the fibre. For ATP which is actively used up by the muscle, and ADP and P_i which are produced, this creates a problem due to the slow diffusion of these ions into and out of the muscle. Without suitable buffering of the ATP, the centre of the fibres can be starved of ATP and have enhanced ADP and P_i concentrations. Experiments in which the ADP concentration is kept low can use a suitable rephosphorylating system such as phosphocreatine and creatine kinase (EC 2.7.3.2), but the problem becomes more tricky when other concentrations of ATP and ADP are needed, even with the use of adenylate kinase (EC 2.7.4.3).

(3) Any suitable solution contains many different ionic species present which have a potential influence upon the kinetics. For example, in the solutions used for the experiments described in this paper, we have added the following ions: K^+, Na^+, Mg^{2+}, Ca^{2+}, Cl^-, ATP^{4-}, ADP^{3-}, PO_4^{3-}, histidine$^-$, $EGTA^{4-}$, phosphocreatine$^-$ and H^+. Many complexes are formed between these ions. Those for which we have some idea of the dissociation constants are shown in Table 1. Others (alone or together) could be crucial either in their own right or because they mop up the pool of free ions of one sort or another. We evaluated the concentrations of complexes quoted here from the amounts of the individual ions added and the dissociation constants of Table 1, using a suitable computer iteration procedure (Perrin & Sayce 1967). When phosphocreatine has been added we have assumed a pK of 3 for the association of this with magnesium.

We report the results of mechanical experiments on glycerol-extracted fibres from the dorsal longitudinal muscle of the giant water bug, *Lethocerus cordofanus*. A full description of the apparatus and procedures is given by White & Thorson (1972). We attach one or more fibres of the muscle between two points, one of which is the moving arm of an electromechanical loudspeaker vibrator and the other is attached to a tension transducer. Length changes with a rise time of 1 ms can be applied to the muscle, and the frequency response of the tension transducer enables tension recordings to be made within this

TABLE 1

Ionic species and association constants

Ionic species	$log_{10} K^a$	*Source*	*Ionic species*	$log_{10} K$	*Source*
Hhistidine	9.16	[b]	MgATP	4.0	[b]
H_2histidine	15.22	[b]	MgHATP	8.62	[b]
H_3histidine	17.04	[b]	CaATP	3.6	[b]
Mghistidine	2.1	[b]	CaHATP	8.46	[b]
Cahistidine	1.4	[b]	HADP	6.35	[b]
HEGTA	9.46	[c]	H_2ADP	10.34	[b]
H_2EGTA	18.31	[c]	CaADP	2.78	[b]
H_3EGTA	20.99	[c]	MgADP	3.11	[b]
H_4EGTA	22.99	[c]	MgHADP	7.87	[b]
MgEGTA	5.21	[c]	KADP	1.15	ATP value
MgHEGTA	12.22	[c]	NaADP	1.17	ATP value
CaEGTA	11.00	[c]	HPO_4	12.36	[b]
CaHEGTA	14.18	[c]	H_2PO_4	19.56	[b]
KEGTA	0.96	EDTA value	H_3PO_4	21.68	[b]
NaEGTA	1.8	EDTA value	$KHPO_4$	12.81	[b]
HATP	6.5	[b]	$NaHPO_4$	12.91	[b]
H_2ATP	10.55	[b]	$MgHPO_4$	14.86	[b]
KATP	1.15	[b]	$CaHPO_4$	14.56	[b]
NaATP	1.17	[b]			

[a] K = total association constant (i.e., for H_3histidine, $K = [H_3\text{histidine}]/[H]^3 \cdot [\text{histidine}]$) with dimensions $l^n\ mol^{-n}$ (where n is an integer).
[b] Sillen & Martell (1964). [c] Portzehl *et al.* (1964).

time scale. The fibres are immersed in solutions inside a temperature-controlled bath. All experiments reported here were done at 20 °C. The input to the servomechanism controlling the vibrator position is an analogue output from a minicomputer. This enables any desired waveform to be obtained.

A full discussion of the properties and our selection of this insect fibrillar flight muscle for experiments is given elsewhere (White & Thorson 1973). The obvious difference between it and vertebrate striated muscle is that small length changes produce large differences in equilibrium tension and ATPase activity: an increase of length of 1% above rest length in the active muscle causes a 100% increase in the rate of hydrolysis of ATP by the muscle (Rüegg & Tregear 1966). Such a length change also produces large maintained increases in the active tension (e.g. see Fig. 2). Probably, these effects are due to the modification of one or more of the rate constants of the cross-bridge cycle by alterations of the muscle length. If so, it becomes a powerful ally in the study of the cycle. In the simplest explanation of this strain activation (Thorson & White 1969) it is suggested that an increase of length causes an increase in the rate constant of attachment. The presence of inorganic orthophosphate ions

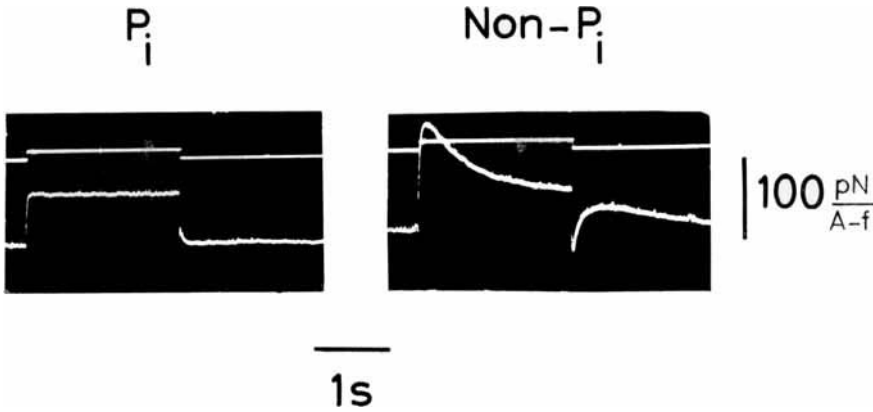

FIG. 2. Effect of a step change of length of 0.5% on *Lethocerus cordofanus* muscle in (left) the presence of 20mM-orthophosphate (P_i) and (right) the absence of phosphate (non-P_i). The constituents of the solutions are specified in Table 2.

caused a dramatic modification of the response obtained in the absence of P_i (White & Thorson 1972) (see Fig. 2). The free Mg^{2+} and MgATP concentrations and the ionic strength were virtually the same in both solutions. The difference seems to be a direct effect of inorganic orthophosphate. In the

TABLE 2

Solutions used in experiments. The P_i and non-P_i solutions refer to Fig. 2, solutions 1–9 to Fig. 3: 1–3 top row, left to right; 4–6 the middle and 7–9 the bottom row. All concentrations (except Ca^{2+}) are in mmol/l. The MgATP, Mg^{2+} and Ca^{2+} concentrations are calculated as described in the text.

	P_i	*Non-P_i*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Na_2ATP	10	10	11	11	12	2.2	2.2	3.0	0.5	0.55	0.8
$MgCl_2$	12	10	20	13	10.5	11	4.5	3.0	10	3.5	1.5
EGTA	5	5	5	5	5	5	5	5	5	5	5
$CaCl_2$	3	3	5	5	5	5	5	5	5	5	5
KCl	20	45	25	35	35	50	65	60	50	65	70
K_2HPO_4	13	0	0	0	0	0	0	0	0	0	0
KH_2PO_4	7	0	0	0	0	0	0	0	0	0	0
Histidine HCl	0	20	20	20	20	20	20	20	20	20	20
NaCl	0	0	0	0	0	16	16	16	20	20	20
KOH	0	0	34	35	32	25	20	20	20	16	20
pH	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Ionic strength	118	117	134	129	129	131	130	125	128	128	132
MgATP	8.34	8.55	10.7	9.8	9.0	2.1	1.9	1.6	0.48	0.48	0.50
Mg^{2+}	1.20	1.35	7.8	2.2	0.8	7.4	1.7	0.5	8.1	2.0	0.5
$Ca^{2+}/\mu mol\ l^{-1}$	0.32	0.32	30	21	15	32	31	23	32	32	31

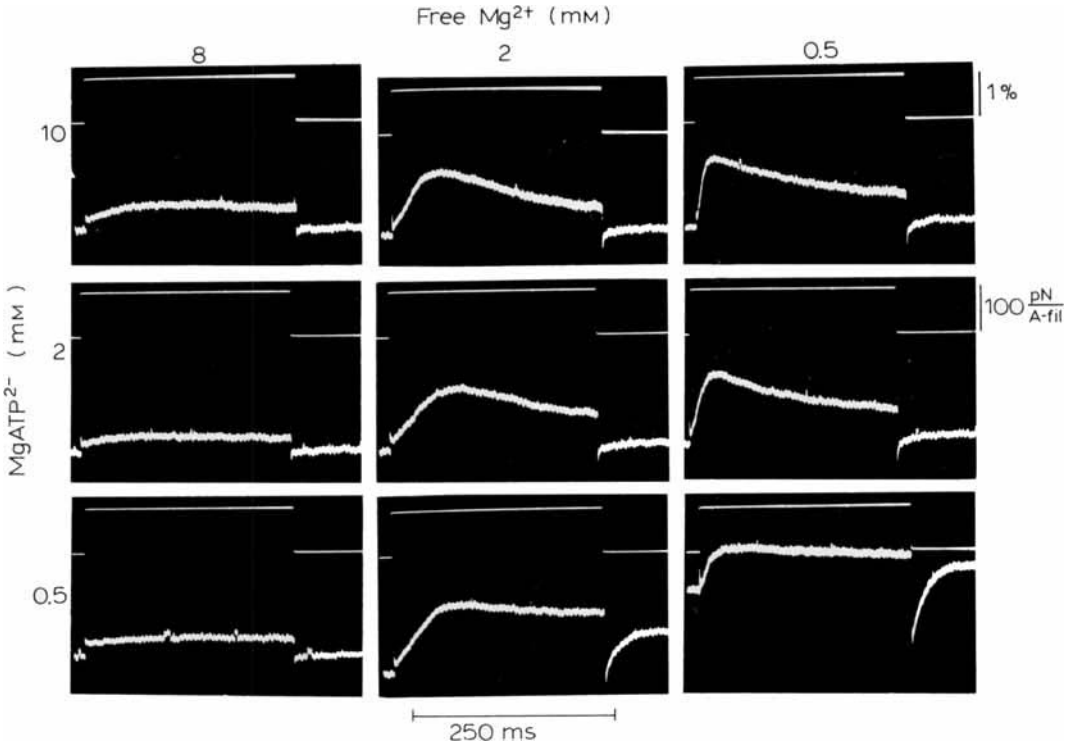

Fig. 3. Response of *Lethocerus cordofanus* muscle to a 1% step change of length in the presence of the concentrations of Mg^{2+} and $MgATP^{2-}$ indicated. The full constitution of the solutions is given in Table 2. The responses in 0.5mM-$MgATP^{2-}$ would ideally have continued for rather longer; the tension finally returns to its initial value. In the 2mM-Mg^{2+} responses, the rate of rise of tension at 0.5mM-$MgATP^{2-}$ is faster than that in 2mM-$MgATP^{2-}$. When alternating between these two solutions this was not the case.

absence of P_i the tension rises to a high value and then decays to its new equilibrium value after a step increase of length. This overshoot, which we called a phosphate starvation transient, disappears when the phosphate concentration exceeds about 5 mmol/l.

We have recently been investigating the effect of MgATP concentration on the mechanical response. $MgATP^{2-}$ (rather than ATP^{4-}) is the substrate for the ATPase site of myosin (Finlayson *et al.* 1969). In preliminary experiments, we adjusted the concentration of MgATP by simply changing the concentration of ATP, keeping the added $MgCl_2$ constant at 10 mmol/l. This caused large changes in the concentration of free Mg^{2+} in the final solution also. We were surprised to discover in control experiments that free Mg^{2+} markedly affects

the dynamics of the muscle. Fig. 3 illustrates the responses obtained in solutions over a range of Mg^{2+} and $MgATP^{2-}$ concentrations, in the absence of P_i.

The effect of changing the free Mg^{2+} concentration from 0.5 to 8 mmol/l is distinctive. The consequence of a change in the fibre length, after an initial spike (not clearly shown in these figures), is a delayed increase in tension. The rate of rise of tension (high at low Mg^{2+} concentrations) is considerably reduced as the Mg^{2+} concentration is raised. The phosphate starvation transient is no longer apparent at 8mM-Mg^{2+}. This general trend is present at all three MgATP concentrations studied here.

The effect of changing the MgATP concentration at constant Mg^{2+} is less dramatic. The rate of rise of the delayed tension is slightly greater at the higher MgATP concentrations. The greatest effect, however, is in the subsequent decay of tension. At the lowest MgATP concentration we used the tension falls little from its peak value. As the length is reduced to its initial value, the tension falls a lot initially at 0.5mM-MgATP, before increasing transitorily to a large extent, similar to a phosphate starvation transient. At the higher MgATP concentrations the initial fall of tension is much smaller, and little delayed rise of tension is apparent. These effects are not seen at 8mM-Mg^{2+} at which concentration the magnitude of the delayed tension change is greater at greater MgATP concentrations. Unfortunately, our tension transducer was not stable enough to provide accurate measurements of zero tension. For all except the response in 0.5mM-Mg^{2+} and 0.5mM-ATP the baseline lay about 30–50 pN/(A filament) below the initial tension. The initial tension in the 0.5mM-Mg^{2+} and 0.5mM-ATP response was about 200 pN/(A filament).

As discussed earlier, one objective is the understanding of these results in terms of a testable model of cross-bridge activity. One method of building such a model might be to assume that each of the biochemist's states has its mechanical counterpart, and to build a multistate mechanical cycle. With the scheme of Fig. 1, this would result in a seven-state cycle in which there were five attached states each of which would provide its own contribution to the tension, and the rate constants between each of which would depend on cross-bridge distortion. This results in a model with too many unknown parameters to be useful. An alternative approach is to ask what is the simplest model capable of explaining the results and to see if experiments can be devised to show its incorrectness.

Previous work in this context to account for the properties of *Lethocerus* muscle has been summarized elsewhere (White & Thorson 1973). Essentially, a three-state model is adequate to explain the responses obtained in 8mM-MgATP and 2mM-Mg^{2+} in both the presence and absence of P_i. On this model (Fig. 4) the effect of P_i was assumed to be an increase in both the backward and forward

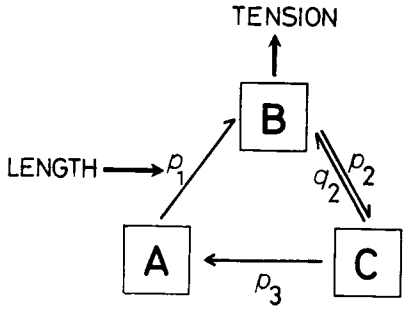

FIG. 4. Three-state cycle of cross-bridge activity used to explain the responses in the presence and absence of phosphate (White 1972). The backward rate constants of steps 1 and 3 are assumed to be negligibly small: p_1 is assumed to be proportional to fibre length, and the tension is a function of the number of cross-bridges in state B and of their distortion.

rate constants between states B and C. When these rates constants are much greater than those between states A and B and between states C and A the model becomes essentially a two-state model. The phosphate starvation transient is explained in the following way. At the low length, before the step, the attachment rate constant p_1 is the lowest and state A is thus the most populated state. Increasing the length increases the value of p_1. At equilibrium, state C is now much more highly populated than before, but in reaching this equilibrium state B becomes temporarily over-populated, thus causing a large tension overshoot. Phosphate, by increasing the rate constants between states B and C, prevents this temporary overpopulation of state B. Numerous other properties of the muscle are also explained fairly readily in terms of this model. Its unattractive feature is that state C is a non-tension producing state, and there is no good reason, from other results, to suggest that P_i should increase the forward rate constant p_2. That P_i might increase the backward rate constant q_2 can be understood if this step is that at which P_i is released (step 3 of Fig. 1).

This model predicts the stiffness of the muscle at different stages of the phosphate starvation transient: the stiffness should be proportional to the number of cross-bridges in state B. There should thus be an increase in stiffness with increasing tension, contrary to the results of Schädler *et al.* (1971). Such an experiment is shown in Fig. 5 in which a second small (0.3%) test step has been given at various times after the initial applied length change. Fig. 6 shows the resulting stiffness measurements from these and other traces from the same experiment. The stiffness changes with tension, but there is no detectable difference in the stiffness at a given tension in the rising phase of tension as compared to that at the same tension in the falling phase of tension in the phosphate starvation transient.

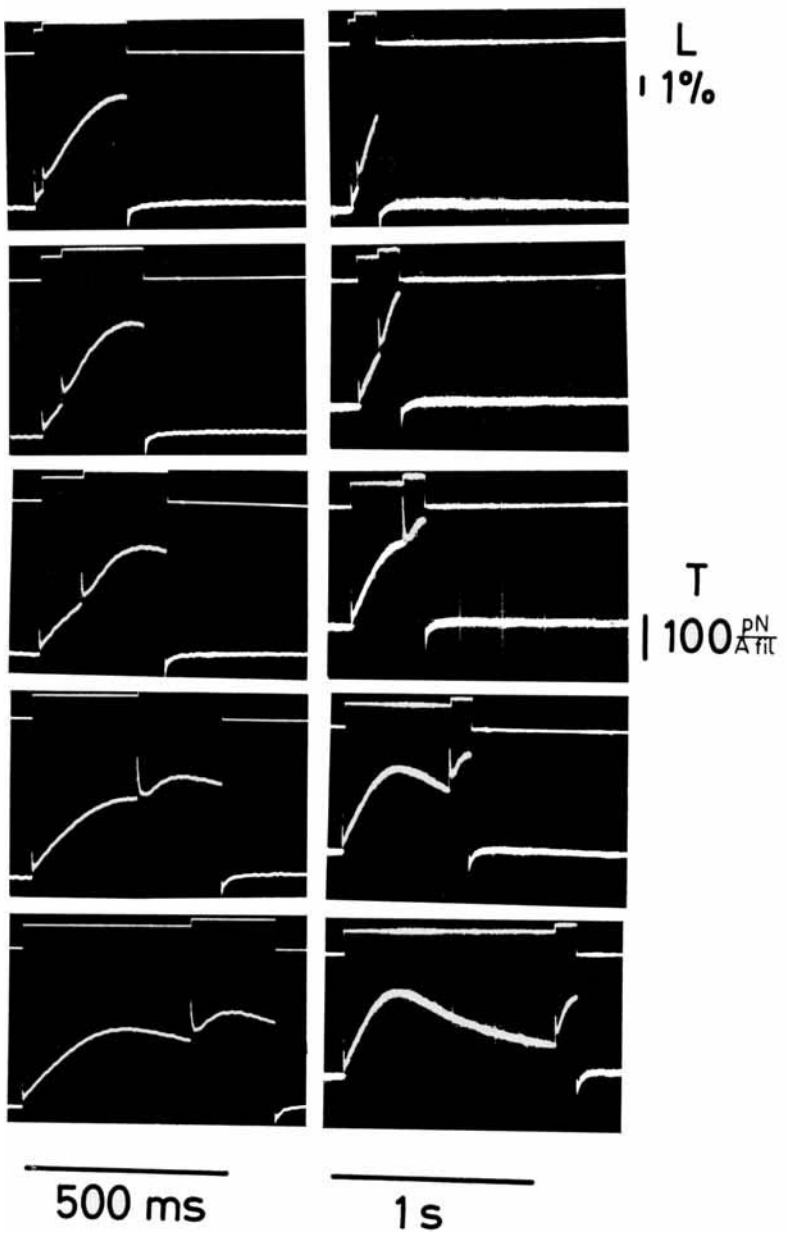

FIG. 5. Response of *Lethocerus cordofanus* muscle to a step increase of length of 1% followed by an increase of 0.3% at various times afterwards. The magnitude of the immediate response to the 0.3% length change is greater the greater the tension in the muscle at which it was applied.

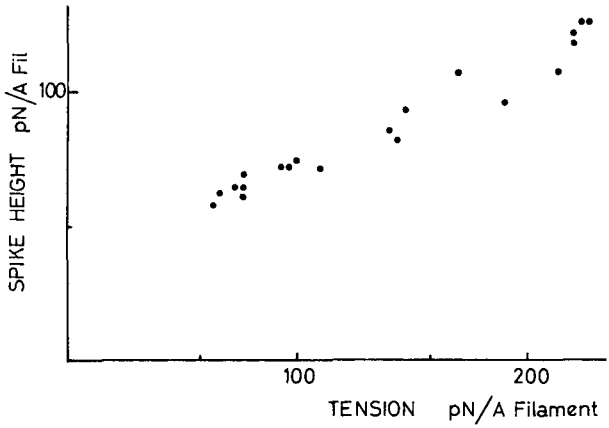

FIG. 6. Spike height of the response to the 0.3% length change, in the experiments of Fig. 5, as a function of the tension in the muscle when the step length change was applied. The responses measured from several other traces from this experiment, not included in Fig. 5, are included.

At the moment we cannot explain simply how the effects of varying Mg^{2+} and MgATP are produced. Although, in general terms, MgATP increases (1) the initial rate of rise of tension in the experiments we have described and (2) the magnitude of the fall of tension from the peak value obtained in the phosphate starvation transient, both of which can separately and qualitatively be explained by an increase in a forward rate constant, we have yet to produce a satisfactory three- or four-state model in which these effects are brought about simply by changing one forward rate constant between two states. We do not feel that explanations in terms of a multiplicity of effects of MgATP on rate constants are sufficiently simple to be worth publishing at this stage.

In conclusion, our purpose has been to demonstrate a way of attacking the problem of the link between the biochemical and mechanical kinetics. Further work of this kind, together with tests of the state of the muscle at different times by double-step experiments should enable a number of candidate explanations of the cross-bridge cycle to be tested.

ACKNOWLEDGEMENTS

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Discussion

Hess: Could you follow the transient by a determination of the extent of the reaction using labelling experiments? Also, wouldn't it be better to let the muscle oscillate, because a phase analysis of the oscillating chemical species might supply much more information?

White: For small signal experiments in which the muscle behaves linearly—that means that the response to a decrease of length is the inverse of the response to an increase in length—oscillatory methods represent a good experimental approach. However, with the amplitudes we use, with which we observe non-linearities, we do not have simple phase-shift problems any more: the wave-forms from the muscles are complicated, for example, figures of eight (see e.g. White & Thorson 1973). The mechanisms giving rise to such responses are much more difficult to understand by sinusoidal analysis than by transient analysis (i.e. applying step changes of length).

We have analysed the oscillatory response as well. Our model enables us to provide a reasonable explanation of the response obtained to both a sinusoidal

input of length at different frequencies and to a step change of length. Formally, the two methods are equivalent. My point is that the transient analysis is the easier to interpret.

Wilkie: You seem to have a bewildering selection of variables. How will you avoid getting completely entangled in them?

White: To some extent, we are a little entangled in them now! However, we expect that the more responses we analyse, the simpler it will be to envisage the overall cycle that we shall have to develop. We shall have to apply more constraints for the more different kinds of response we obtain. A major problem is the number of control experiments we have to do: for example, we once thought we were studying ATP concentrations only to find we were really following the concentration of magnesium ions. Our worst trouble is that most association constants we obtain from sources such as *Stability Constants* (Sillen & Martell 1964); the constants obtainable are not always determined in the same ionic strength or at the same temperature.

Cohn: Also, those values now seem rather outmoded. George *et al.* (1963) have published association constants as functions of ionic strength, temperature and so on, that are far better than those of Sillen & Martell.

Woledge: Others you could measure. For example, a magnesium electrode is available which might give better values for the free magnesium ion concentration than you will find in the literature.

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Protein phosphorylation and metabolic control

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Abstract The regulation of enzyme activity through the phosphorylation and dephosphorylation of specific seryl or threonyl residues in enzymes is now recognized as an important control mechanism. A great many non-enzymic proteins may also be interconverted between phosphorylated and non-phosphorylated forms, but in these instances the function served by phosphorylation is not well understood. This lack of understanding is probably due to our lack of knowledge of the specialized actions of most non-enzymic proteins.

The sequences of phosphorylation and dephosphorylation of proteins are, of necessity, subject to rigid control. If this were not so, the ATP supply of a cell would be rapidly depleted. Moreover, such processes must be regulated for metabolic interconversions to have a physiological regulatory role. For the best studied system, the interconversion of phosphorylase *b* and phosphorylase *a*, many different factors controlling the phosphorylation and dephosphorylation steps have been elucidated. Probably this process is constrained so that it uses only a little energy. In this paper, these constraints are examined.

The regulation of enzymic activity through the phosphorylation and dephosphorylation of specific seryl or threonyl residues in the enzyme is now recognized as an important control in metabolism. Such a sequence of phosphorylation and dephosphorylation, as illustrated for the general case in equations (1) and (2), should be distinguished from the formation and breakdown of the intermediary phosphoenzyme complexes in enzyme reactions (or transport mech-

$$\text{Enzyme} + n\text{ATP} \xrightarrow{\text{protein kinase}} \text{Enzyme-P}_n + n\text{ADP} \qquad (1)$$

$$\text{Enzyme-P}_n + n\text{H}_2\text{O} \xrightarrow[\text{phosphoprotein phosphatase}]{} \text{Enzyme} + n\text{P}_\text{i} \qquad (2)$$

anisms), in which only one enzyme is directly involved, in contrast to the three in the phosphorylation and dephosphorylations that are of interest here. The entire system consists of the protein substrate, a protein kinase and a phosphoprotein phosphatase; together, the system constitutes an ATPase.

The best documented examples of metabolic control through the reversible phosphorylation of enzymes are found in bioenergetic pathways, particularly those concerned with the biosynthesis and degradation of energy-storage compounds (Table 1 *A*). This type of control mechanism uses ATP and thus drains off a portion of the energy generated or being stored in the particular pathway. The phosphorylation and dephosphorylation of enzymes represents a potential 'futile cycle' (Scrutton & Utter 1968). It is fitting that these systems be explored in a symposium concerned with energy transformations.

TABLE 1

Systems for the phosphorylation and dephosphorylation of proteins

	EC Number		
(A) Enzymes		*(B)* Non-enzymic proteins	
Phosphorylase	2.4.1.1	Histones and protamines	
Phosphorylase kinase	2.7.1.38	Non-histone nuclear proteins	
Glycogen synthetase		Ribosomal proteins	
Hormone-sensitive lipase		Myofibrillar proteins	
Pyruvate dehydrogenase		actin	
Pepsinogen		troponin (TN-I, TN-T)	
Cyclic AMP-dependent protein kinase[a]		light myosin chain	
		Connective-tissue proteins (collagen, etc.)	
		Membrane proteins	
		proteins of cell membrane	
		proteins of sarcoplasmic reticulum	
		rhodopsin	
		Neurotubular proteins	
		Calcium-binding protein of brain	

[a] The regulatory subunit of the cyclic AMP-dependent protein kinase of bovine heart is phosphorylated (Rosen *et al.* 1973); the analogous enzyme from skeletal muscle, as studied in our laboratory (J. A. Beavo, P. J. Bechtel & E. G. Krebs, unpublished work), does not undergo rapid phosphorylation.

We shall consider the most intensively studied of the several known sequences of phosphorylation and dephosphorylation, namely, the skeletal muscle glycogen phosphorylase system (EC 2.4.1.1) (Krebs & Fischer 1962; Fischer *et al.* 1971; Graves & Wang 1972). We shall also deal briefly with phosphorylations of proteins other than enzymes.

General aspects

Phosphorylase, which catalyses the first step in glycogenolysis, is considered to be the major regulatory enzyme in this pathway. The enzyme is regulated by metabolites (i.e. allosteric control) as well as by phosphorylation (see equations 3 and 4).

$$\text{Phosphorylase } b + 2\text{ATP} \xrightarrow[\text{kinase}]{\text{phosphorylase}} \text{Phosphorylase } a + 2\text{ADP} \quad (3)$$

$$\text{Phosphorylase } a + 2\text{H}_2\text{O} \xrightarrow[\text{phosphatase}]{\text{phosphorylase}} \text{Phosphorylase } b + 2\text{P}_i \quad (4)$$

Phosphorylase *b*, the unphosphorylated form of the enzyme, is essentially inactive at finite substrate concentrations unless AMP is present, but phosphorylase *a*, the phosphorylated form, is almost fully active in the absence of any cofactor. Although there may be special circumstances under which the activation of phosphorylase *b* by AMP is physiologically significant, the major means of activating the enzyme is its conversion into the *a* form.

Phosphorylase *b* is converted into phosphorylase *a* when muscle contracts in response to electrical stimulation (Cori 1956) and also to stimulation by adrenaline (Sutherland 1951). In resting muscle, the enzyme exists almost entirely in the dephospho or *b* form (Krebs & Fischer 1955). As the relative amount of phosphorylase *a* or *b* present at any time is governed by the respective rates of reaction of phosphorylase kinase and phosphorylase phosphatase, these steps must be regulated in order to account for the variations in amount of *a* and *b*.

Phosphorylase kinase

Phosphorylase kinase is a relatively specific protein kinase that catalyses the phosphorylation of a single seryl residue (Ser-14, in bold type in sequence below) near the NH_2-terminus of phosphorylase (personal communication from E. H. Fischer):

NAc-Ser-Arg-Pro-Leu-Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-
Ser-Val-Arg-Gly-Leu-Ala-Gly-Val-Glu- · · ·

The only other protein which this kinase is known to phosphorylate at an appreciable rate is the inhibitory subunit of troponin, TN-I (Stull *et al.* 1972).

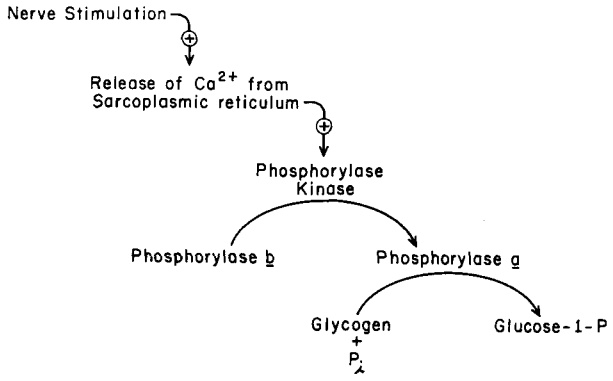

FIG. 1. The regulation of glycogenolysis by calcium ions (reproduced by permission of Medical and Technical Publishing Co., Ltd.)

From a regulatory standpoint, the enzyme possesses several properties which suggest that it is actively controlled within the cell.

Phosphorylase kinase from skeletal muscle exhibits a mandatory requirement for Ca^{2+} (Meyer *et al.* 1964). The enzyme is sensitive to the metal ion with half-maximal activation at about 0.2μM-Ca^{2+} (Ozawa *et al.* 1967), a concentration which is slightly lower than that required for the development of maximal contractile tension in muscle. It is generally believed that the release of Ca^{2+} from the sarcoplasmic reticulum provides the link whereby muscle excitation is coupled to contraction and glycogenolysis. The cascade system in the latter process is illustrated in Fig. 1. The sarcoplasmic reticulum fraction from skeletal muscle can completely block the conversion of phosphorylase *b* into phosphorylase *a*.

A second property of phosphorylase kinase that is of major importance to the control of its activity is that, like phosphorylase itself, it is phosphorylated and dephosphorylated (Krebs *et al.* 1966). As with phosphorylase, phosphorylation enhances its activity. Activated (phosphorylated) and non-activated phosphorylase kinase both require Ca^{2+}, but the former is more sensitive to the metal ion than the latter form. The activation of phosphorylase kinase is catalysed by a second protein kinase that requires cyclic AMP for activity (Walsh *et al.* 1968) and is referred to as a cyclic AMP-dependent protein kinase rather than by the more restrictive name 'phosphorylase kinase kinase', because it operates on a broad range of substrates. Activation of phosphorylase kinase by the cyclic AMP-dependent protein kinase is believed to constitute the mechanism whereby adrenaline and other hormones promote the conversion

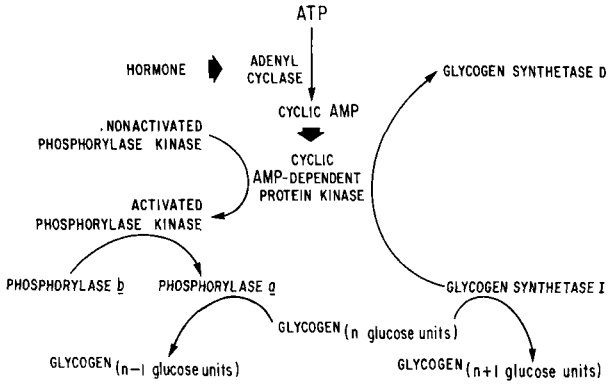

FIG. 2. The regulation of glycogenolysis and glycogenesis by the cyclic AMP-dependent protein kinase (reproduced by permission of the American Society of Biological Chemistry, Inc.)

of phosphorylase *b* into phosphorylase *a* (Fig. 2). This same kinase catalyses the phosphorylation and inactivation of glycogen synthetase.

Another potential means of regulating phosphorylase kinase activity is through its interaction with glycogen (DeLange *et al.* 1968). Glycogen is a strong activator of the enzyme and may well promote its own degradation when its concentration reaches a certain level in muscle, by causing phosphorylase *a* formation. Glycogen can also regulate its own biosynthesis through its inhibitory effect on glycogen synthetase phosphatase (Larner & Villar-Palasi 1971), the enzyme that promotes the dephosphorylation and activation of glycogen synthetase. Larner & Villar-Palasi have noted that the uppermost glycogen concentration in muscle appears to be 'tightly controlled' at about the 1% level.

Finally, the activity of phosphorylase kinase is strongly depressed when the concentration of ATP exceeds that of Mg^{2+} (Krebs *et al.* 1964). This may be due to inhibition of the enzyme by ATP or to a requirement for free Mg^{2+}. The possible physiological significance of this type of regulation has been stressed by Villar-Palasi & Wei (1970).

Phosphorylase phosphatase

Of the two enzymes controlling the cycle of phosphorylation and dephosphorylation of phosphorylase (equations 3 and 4), the inactivating enzyme, phosphorylase phosphatase, has received less attention than phosphorylase kinase. It seems probable, however, that the cycle is regulated by both enzymes,

and an examination of the properties of the phosphatase suggests that this is so. The enzyme is strongly inhibited by AMP (Sutherland 1951), an effect that is mediated through the interaction of the nucleotide with the substrate of the phosphatase (i.e. phosphorylase *a*). The efficacy of AMP as a regulator of the phosphatase is shown by the fact that in a model system containing phosphorylase *a*, AMP, and phosphorylase phosphatase, no phosphorylase *a* was converted into phosphorylase *b* until AMP deaminase (EC 3.5.4.6) was added (Krebs *et al.* 1964). Phosphorylase phosphatase is also inhibited by glucose 1-phosphate and inorganic phosphate (Cori & Cori 1945). The enzyme is activated by bivalent cations and by glucose or glucose 6-phosphate. Martensen *et al.* (1973) have extensively studied whether these latter effectors influence the phosphatase reaction as a result of interaction with the enzyme or with substrate.

The inhibition or activation of phosphorylase phosphatase has mostly been investigated with purified phosphorylase phosphatase and crystalline phosphorylase *a* as the substrate. That this approach may be misleading, or may result in a failure to detect phenomena that occur in the intact cell, is suggested by the work of E. H. Fischer and his collaborators who studied what they referred to as the 'glycogen particle'. This consists of a glycogen–enzyme complex that contains all the enzymes of the phosphorylase system and, in addition, the sarcoplasmic reticulum. Using this preparation, they determined that phosphorylase phosphatase subsists under constraints as a result of its interaction with other macromolecular components of the system. The enzyme is reversibly inhibited when the activation of phosphorylase is triggered by Ca^{2+} and ATP. This inhibition is not due to AMP (Haschke *et al.* 1970).

Overview of the system

If the components of the phosphorylase phosphorylation–dephosphorylation cycle and its regulatory factors are considered as a whole (Fig. 3), it is apparent that the system does not operate as an unfettered ATPase. The phosphorylase kinase and the phosphorylase phosphatase reactions are each subject to elaborate controls. In a given set of physiological conditions the balance shifts so that either phosphorylase *a* or phosphorylase *b* will predominate, but there is no indication that appreciable recycling is necessary for these shifts.

On a quantitative basis, the phosphorylation and dephosphorylation of phosphorylase alone would probably not be of major importance in the total economy of the muscle cell even if the cycle were unrestrained. Gratecos *et al.* (1973) have calculated that these reactions could account for the use of less than 1% of the total muscle ATP/min. As the complete turnover of ATP in

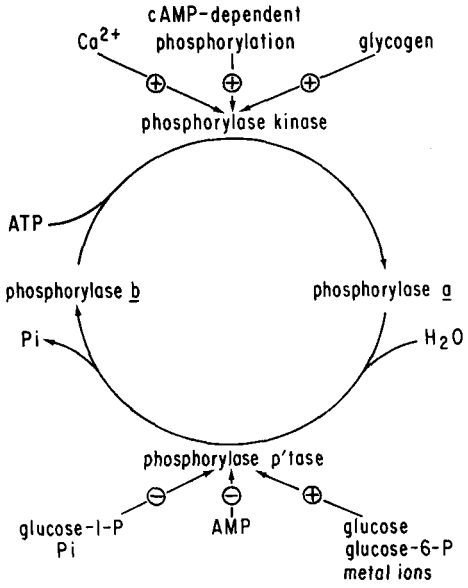

FIG. 3. Factors influencing the phosphorylation–dephosphorylation cycle of glycogen phosphorylase.

resting muscle requires about five minutes, this means that the phosphorylase cycle might consume at most 5% of the total ATP that is being used in these conditions. With maximal work, the use of ATP by muscle rises several hundred-fold and the fraction of the total that could be accounted for by the phosphorylation and dephosphorylation of phosphorylase would be extremely small. We should bear in mind, however, that phosphorylase is only one of several enzymes that can be phosphorylated (see Table 1). Moreover, as we shall now point out, some non-enzymic proteins are also phosphorylated and dephosphorylated.

NON-ENZYMIC PROTEIN SUBSTRATES

In addition to the enzymes that exist in phosphorylated and unphosphorylated forms, numerous other cellular proteins that are phosphorylated have been recognized in recent years. A representative but not necessarily exhaustive list of such proteins is presented in Table 1*B*. It is not known whether phosphorylated and nonphosphorylated forms are necessarily interconverted *in vivo* for all these proteins, but probably this is so in most instances. Investigators have successfully demonstrated the presence of protein kinases, which catalyse

the phosphorylation of these proteins, and have detected appropriate phosphoprotein phosphatases also. Unlike the picture presented for the phosphorylation and dephosphorylation of enzymes, however, it has seldom been possible to define the function of the phosphorylation of these proteins. Reasons for this lack of understanding are related in turn to our lack of knowledge about the function of most non-enzymic proteins.

In the present context, we ought to consider the likelihood of rapid recycling of the sequence of phosphorylation and dephosphorylation for the non-enzymic proteins. Considered as a whole, they are more abundant than the phosphorylated enzymes and, thus, may constitute a more important substrate for ATPase systems of this type. We can expect, however, that mechanisms for controlling the rates of phosphorylation and dephosphorylation of these proteins will also be elucidated.

ACKNOWLEDGEMENTS

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Discussion

Glynn: Is it generally true that, for control, enzymes are phosphorylated on their seryl residues?

E. Krebs: Either seryl or threonyl residues are phosphorylated.

Roseman: Jencks would probably refute that. He maintains that we can never be sure of the location of the phosphoryl group because of its ability to migrate. He has questioned the evidence for its linkage to histidine in phosphoHPr. For example, in phosphoimidazoles, such as phosphohistidine, the phosphoryl group migrates spontaneously from the N-1 to the N-3 atom through an intermediate N^1N^3-diphosphate.

Huxley: But there the phosphate only migrates round one histidyl residue.

Roseman: That was a simple example. His point is that the final observed position of the group, for example on serine, may not be the same as its origin.

Lipmann: Boyer's protein, for example, has phosphate bound to histidine and this is alkali-stable but acid-unstable. However, all the seryl- or threonyl-bound phosphates are alkali-labile and acid-stable.

E. Krebs: Also, from phosphorylase one can get a tetradecapeptide which

contains no histidine and yet can be phosphorylated by phosphorylase kinase in the right position.

Hagins: For those of us who are not experts on phosphorylation, could you list the criteria we ought to apply in evaluating the functional significance of an observation that a particular protein can be phosphorylated?

E. Krebs: Before a given enzymic protein phosphorylation can be accepted as physiologically significant, several criteria should be applied. Does phosphorylation of the protein change a demonstrable function? Is the change reversed by dephosphorylation? Does phosphorylation occur in the intact cell? It is *not* sufficient simply to demonstrate that a protein can be phosphorylated *in vitro* with a protein kinase as the catalyst.

Weis-Fogh: The living muscle contains a fine control system concerning the glycolytic cycle. Cyclic AMP also affects lipase in a similar way. Lipid metabolism in ordinary aerobic conditions is much more important for the living animal than carbohydrate metabolism. Have the regulatory processes been worked out to the same detail for the lipase system as for the carbohydrate system?

E. Krebs: The lipase has not been studied to the same extent. In crude preparations (fat cell homogenates or partially purified fat cell preparations), the lipase is activated by a cyclic AMP-dependent protein kinase that requires ATP and it is assumed that the lipase is phosphorylated. Steinberg has obtained some direct evidence for this.

Hess: Would you like to comment about the regulatory subunit of the cyclic AMP-dependent protein kinases?

E. Krebs: Rosen *et al.* (1973) have shown for the protein kinase from heart muscle that the regulatory subunit is rapidly phosphorylated by the catalytic subunit. This does not occur with the skeletal muscle enzyme that we are studying.

Gutfreund: Which of the kinases phosphorylates the myosin chain in Perry's experiments (Perrie *et al.* 1973)?

E. Krebs: Perry has demonstrated a protein kinase that is distinct from the cyclic AMP-dependent protein kinase and phosphorylase kinase.

Gutfreund: In the cyclic AMP-dependent protein kinase, which like most kinases uses MgATP as a substrate, the MgATP is also required to accelerate the formation of the inhibited complex. How does the balance between ATP and cyclic AMP tie up with the physiological concentrations in the muscle?

E. Krebs: We don't understand the system completely. The binding of the catalytic subunit, C, by MgATP should favour dissociation of R_2C_2. But the fact that MgATP also binds to R_2C_2 with high affinity keeps this substrate

from activating the enzyme. The concentration of MgATP in the cell is such that the enzyme will always be saturated with the complex.

Gutfreund: No thermodynamic problem arises. In the absence of cyclic AMP, ATP accelerates the formation of inhibited complex rather than making it more stable in the presence of cyclic AMP. The inactive tetramer, R_2C_2, dissociates on addition of cyclic AMP (equation 1). Removal of cyclic AMP

$$R_2C_2 \underset{(-cAMP)\ \text{cyclase}}{\overset{+cAMP}{\rightleftharpoons}} R_2(cAMP)_2 + 2C \qquad (1)$$

by diesterase should cause inactivation which should be accelerated by ATP.

E. Krebs: That is because the complex $R_2C_2(MgATP)_2$ is formed. MgATP does not bind tightly to either free R or free C.

Gutfreund: But then the report that it increases the rate of inhibition cannot be correct, for if it only binds to the inhibited complex then it cannot increase the rate of its formation.

Lipmann: ATP may displace cyclic AMP.

Hess: The rate of formation of cyclic AMP constitutes an important step in the regulation of all cyclic AMP dependent functions. What is known about feed-back to the cyclase by 5′-AMP or, in higher organisms, by hormones? Of course, phosphodiesterase exerts also a critical function in maintaining a steady concentration of cyclic AMP. However, from the cases I know, it obviously functions only as a sink without being regulated by additional ligands.

E. Krebs: The diesterase will not attack $R_2(cAMP)_2$; it only attacks free cyclic AMP. The molar concentration of cyclic AMP in the cell is about the same as that of the protein kinase, so that a considerable amount of cellular cyclic AMP is probably always tied up in a form that cannot be attacked by the diesterase.

Huxley: You said that because cyclic AMP does not dialyse off, it cannot be present in the free form, but dialysis is extremely slow compared with dissociation and diffusion over the distances of micrometres. Diffusion is slowed down in proportion to the fraction which is bound at any moment. There will be relatively rapid equilibrium, but diffusion over a few millimetres or a centimetre in dialysis would still be slowed down by a factor of perhaps a million. I wonder if slow dialysis is relevant?

Gutfreund: Again you are considering equilibrium rather than kinetic situations. Even if the binding is tight, ligands can readily be displaced by something else.

Huxley: What is known about the regulation of mitochondrial activity by

either phosphorylation or calcium? Some years ago, Hansford & Chappell (1967) provided evidence that a mitochondrial preparation from insect muscle was powerfully regulated by calcium. They failed to find it in mammalian mitochondria.

E. Krebs: Pyruvate dehydrogenase is regulated by a protein kinase.

Klingenberg: Wieland & Portenhauser (1974) propose regulation through inhibition of the ADP/ATP translocation by acyl CoA. When the lipase is activated, the formation of a large amount of acyl CoA might inhibit the release of ATP from the mitochondria. The internal ATP/ADP ratio regulates the activity of the pyruvate dehydrogenase. Therefore, on increasing the ratio of mitochondrial ATP/ADP by acyl CoA, the ratio of dephosphorylated to phosphorylated pyruvate dehydrogenase decreases.

McClare: If glucokinase is activated by calcium from sarcoplasmic reticulum, could ATP be produced from later steps in glycolysis during a muscle twitch, say, which may be rapid? It would be surprising if the necessary series of reactions were fast enough. Does this mean that the kinase responds to an average calcium concentration over a series of twitches?

E. Krebs: Phosphorylase *a* can be formed by electrical stimulation within less than one second. Certainly, the generation of ATP due to glycogenolysis is far removed, but when the ATP is needed, as in a 100-yard sprint, the muscular contractions are sustained by glycogenolysis.

Huxley: ATP from glycolysis is irrelevant in a single twitch, anyway.

E. Krebs: Previously, one theory proposed that phosphorylase had to be activated with every twitch but that has been disproved.

Taylor: In the lymphocyte system (Zurier *et al.* 1973), in which microtubule polymerization and depolymerization are controlled, both cyclic AMP and cyclic GMP are required. Are any kinases activated by cyclic GMP?

E. Krebs: Kuo & Greengard (1971) have described a cyclic GMP-dependent protein kinase from lobster muscle, but its counterpart in mammalian tissues has not been demonstrated clearly.

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Energy utilization for control

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Abstract When, on addition of a suitable substrate, a chemical potential is applied to an enzymic process such as glycolysis or respiration, whether in solution or membrane-bound, all components of the process pass into a non-equilibrium state, which might be steady or non-steady and which produces the following phenomena:

(1) The reactants of each enzymic reaction are displaced from their equilibrium concentration, and energy is dissipated;

(2) Part of each enzyme is transferred to a transition state of its catalytic function as well as isosteric and allosteric controlling functions, displaying local and gross conformation changes, and a rate-controlling state is generated;

(3) In cyclic portions of a process futile events and chemical interconversion may occur;

(4) In self- and cross-coupled portions of a process, oscillation with periodic changes of states and spatial propagation as well as instabilities may be observed;

(5) At each step of a process, depending on the rate of flux and the specific enzymic function, a varying proportion of the free energy changes—which are concentration-dependent and derived from the overall potential of the system—is contributed to the control of flux rates.

This will be exemplified for enzymes of bioenergetic pathways.

When, by addition of a suitable substrate, a chemical potential is applied to an enzymic process in solution or bound to a membrane, a flux is generated and all components of the process pass into a non-equilibrium state, which might be steady or not. This results in the following phenomena:

(1) the reactants of each enzymic reaction are displaced from their equilibrium concentration and energy is dissipated;

(2) parts of each enzyme or receptor protein are transferred to a transition state of its catalytic function or isosteric and allosteric controlling functions (or both), displaying local and gross changes in conformation—a rate-controlling state is generated;

(3) in cyclic parts of a process futile events and also the chemical interconversion of enzymes and receptor proteins may occur, both yielding rate-controlling functions;

(4) in self- and cross-coupled parts of a process, oscillations with periodic changes of states and spatial propagation as well as instabilities may be observed and

(5) at each step of a process depending on the rate of flux and the specific enzymic function, a varying part of the free energy changes involved, depending on concentration and being derived from the overall potential of the system, is contributed to the control of flux rates.

The minimum energy required to maintain a steady-state flux for a reaction system can be computed from its displacement from equilibrium (Kuhn 1936; Hess 1963; Bücher & Rüssmann 1963; Bücher & Siess 1969). For the reaction $A \rightleftharpoons B$ with rate constants $\vec{k}_A$ and $\overleftarrow{k}_B$, the energy ΔG is given by equation (1), where $\Gamma = [B]_{ss}/[A]_{ss}$, $[A]_{ss}$ and $[B]_{ss}$ are the steady-state concentrations of A and B,

$$\Delta G = RT \ln (\Gamma/K) \tag{1}$$

$K = [B]/[A]$ where [A] and [B] are the concentrations of A and B, respectively, at equilibrium, and $\Gamma/K < 1$ and ΔG is always negative. The amount of energy per unit time, $d\Delta G/dt$, required to maintain a steady-state is thus (2). The

$$\frac{d\Delta G}{dt} = \frac{\Gamma - K}{\Gamma} \cdot \vec{k}_A \cdot [A]_{ss} \cdot RT \ln (\Gamma/K) \tag{2}$$

left-hand side expresses the power, $(\Gamma - K)\Gamma^{-1}\vec{k}_A[A]_{ss}$ is the flux and $RT \ln (\Gamma/K)$ is the potential.

Previously, I have reported experimental values for Γ for the glycolytic reactions in ascites tumour cells (Hess 1963). In general, these data have been confirmed for glycolysis in many other cells. On the basis of these experimental values, glycolytic reactions can be classified into two classes of steady-state behaviour: (1) those with enzymes operating near their thermodynamic equilibrium with a reverse flux being of the order of the forward flux and a low net flux compared to the maximal activity of the enzyme and (2) those with enzymes operating quasi-irreversibly, far from equilibrium, with a reverse flux negligibly small compared to the net flux, and having regulatory functions.

This observation not only prompted the search for regulatory enzymes as control points in glycolysis as well as in other reaction pathways and as the principal source of non-linearity of multi-enzyme systems, but also led to the conclusion that the free energy (as expressed by a very small quotient Γ/K) of enzymes belonging to the second class is used for control. This relation is

illustrated in Table 1, in which, for a given set of experimental conditions drawn from earlier observations (Hess 1963), the range of free energies observed in glycolytic reactions is computed and may be large.

TABLE 1

Relations between Γ/K, $\triangle G$ and the velocities of the reactions.[a]

Γ/K	$\triangle G$/kcal mol^{-1}	v_{-1}/v[b]
1	0	∞
0.5	–0.4	1
0.2	–1.0	0.25
0.1	–1.4	0.11
0.05	–1.9	0.05
0.01	–2.8	0.01
0.001	–4.3	0.001

[a] Adapted from Hess (1963), Hess & Brand (1956) and Rolleston (1972).

[b] Here, $v = v_{+1} - v_{-1}$, where v_{+1} and v_{-1} are defined for the reaction $A \rightleftharpoons B$:

$$v_{+1} = V_f[A]_{ss}/K_s D$$

$$v_{-1} = V_r[B]_{ss}/K_p D$$

where $$D = 1 + [A]_{ss}/K_s + [B]_{ss}/K_p.$$

Hence $$\frac{v_{-1}}{v_{+1}} = \frac{V_r[B]_{ss}K_s}{V_f[A]_{ss}K_p}.$$

Since, here, $[B]_{ss}/[A]_{ss} = \Gamma$ and $K = V_f K_p / V_r K_s$, then

$$\frac{v_{-1}}{v_{+1}} = \frac{\Gamma}{K}$$

and $$\frac{v}{v_{-1}} = \frac{K}{\Gamma} - 1.$$

In the following discussion I shall be concerned with the energy requirement for control of these classes of enzymic reactions. Furthermore, I shall stress what types of dynamic phenomena are generated by this new function of energy use. A discussion of the significance of futile cycles as well as chemical interconversions of enzymes is beyond the frame of reference of this paper; however, every conclusion resulting from the discussions presented here will also apply to the control properties displayed by both phenomena.

'NEAR-EQUILIBRIUM' ENZYMES

The reactions catalysed by 'near-equilibrium' enzymes are driven by a steady-state potential which is indicated by a displacement from equilibrium.

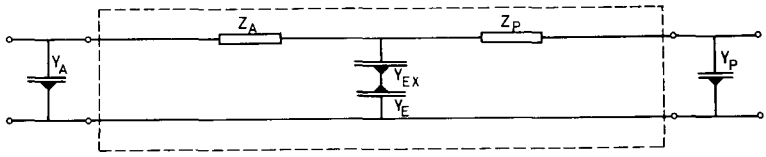

FIG. 1. Isomorphous network of glucosephosphate isomerase (Hess *et al.* 1972). Y, capacitive elements and Z resistive elements for substrate (A) and product (P); EX represents the enzyme–substrate complex, E the free enzyme and Y_{EX} and Y_E its capacitive elements.

The rates of reactions are controlled by the structure of the enzyme, which functions with Michaelis–Menten kinetics. Generally, the substrate concentration in steady-state conditions is well below the Michaelis constant of the enzymes. A typical enzyme of this class is glucosephosphate isomerase (EC 5.3.1.9), which catalyses the reversible isomerization of glucose 6-phosphate into fructose 6-phosphate. The reversibility of the reaction has been demonstrated experimentally in intact yeast cells by its displacement from equilibrium as indicated in the experimental quotient Γ/K (Barwell & Hess 1971).

The topology and dynamic properties of the reaction can be described by an isomorphous chemical network as in Fig. 1 (Hess *et al.* 1972). The network is composed of capacitive elements representing the relation between the chemical potential and concentration of substrate and product as well as the enzyme–substrate intermediate. Furthermore, the resistive elements represent the kinetic rate laws for substrate utilization and product formation and are the parts of the circuit, which dissipate energy. Such a circuit is useful for the division of the elementary subsystems into parts which conserve energy and parts which dissipate energy (Oster & Desoer 1971). With respect to the signal transmission properties, the two-part circuit has a RC-low pass filter property, which might have an interesting function in regard to the operation of the enzyme within the glycolytic pathway.

The relation of the substrate glucose 6-phosphate to the product fructose 6-phosphate in steady-state conditions represented by the network is plotted in Fig. 2 for three different glycolytic fluxes between about 6 mmol l^{-1} min^{-1} up to 33 mmol l^{-1} min^{-1}. Contrasted against the bold line representing the chemical equilibrium of the reaction for a large range of concentrations of substrate and product are the three lines representing the steady-state ratios of product and substrate for the nearly-constant flux conditions. The experiment clearly demonstrates the increasing deviation from the equilibrium line with increasing flux.

A comparison of the values of Γ with the equilibrium constant of the reaction for a given flux yields the free-energy change across the reaction for equilibrium

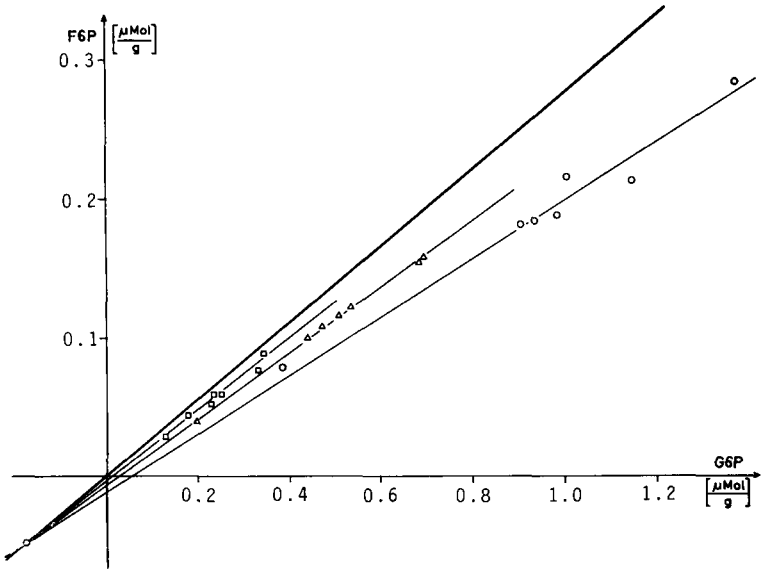

FIG. 2. A plot of the concentration (in μmol/g) of fructose 6-phosphate (F6P) against that of the substrate (G6P) for three flux conditions: 5.7 (□), 13.6 (△) and 32.6 (○) μmol ethanol g^{-1} min^{-1} (from Hess *et al.* 1972); ——, chemical equilibrium of reaction for a large range of concentrations.

(equation 3) and steady-state conditions (equation 4), with a ΔG of –83

$$\Delta G = -RT \ln K = 714 \text{ cal/mol} \quad (3)$$

$$\Delta G = -RT \ln I' = 797 \text{ cal/mol} \quad (4)$$

cal/mol (roughly 10% of the overall ΔG of the reaction) required to maintain the steady state. This amount is due to the high cellular enzyme concentration of glucosephosphate isomerase of roughly 0.1 mmol/l in yeast (Hess *et al.* 1973) which allows only a small deviation from equilibrium for a large physiological flux. Thus, the high concentration, by preserving a near-equilibrium condition, serves to keep the energy for the maintenance of flux as low as possible. In a similar study, the energy expended in the enolase reaction of red and white muscle in its excited state under different flux and temperature conditions was found to be in the range of –560 and –620 cal/mol, respectively (Bücher & Siess 1969).

REGULATORY ENZYMES

Regulatory enzymes catalyse quasi-irreversible reactions in metabolic pathways driven by steady-state potentials across the reaction which may be

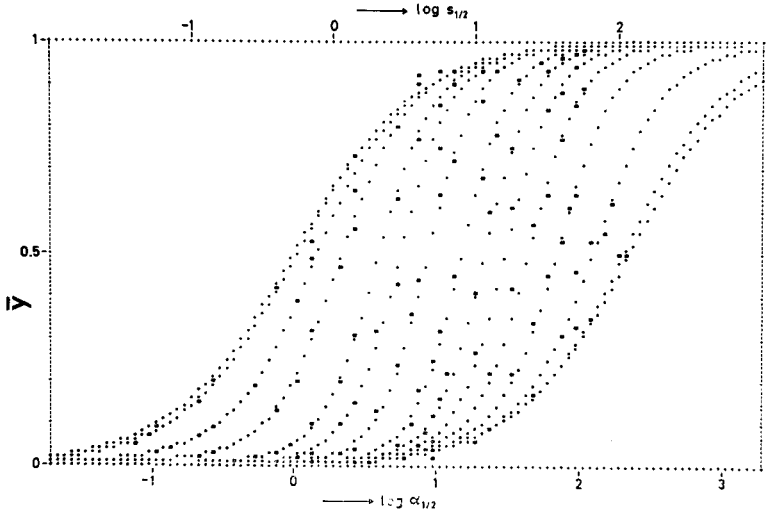

FIG. 3. Computer output of the saturation curves of pyruvate kinase of yeast with limiting curves for the 'R' (left) and 'T' (right) state. The scale on top is given in terms of the substrate concentrations (in l mmol^{-1}), the bottom scale (log $\alpha_{1/2}$) in terms of the normalized substrate concentrations. Closed circles are experimental values, stars are computed values (Johannes & Hess 1973). The ordinate, $\bar{Y}$ is v/V_{max} which is given by

$$\frac{L'c\alpha(1 + c\alpha)^{n-1} + \alpha(1 + \alpha)^{n-1}}{L'(1 + c\alpha)^n + (1 + \alpha)^n}$$

large. Commonly, the enzymes respond non-linearly to concentration changes of the controlling ligands—either substrates and/or products of the reaction (isosteric) or allosteric low molecular structures—by changing their affinities. Usually, the kinetic mechanisms of regulatory enzymes can be described by the model of Monod *et al.* (1965) or of Koshland *et al.* (1966) requiring at minimum a dimeric quaternary structure. The essential feature of oligomeric enzymes is that ligand-induced conformation changes of a monomeric subunit not only might effect the substrate affinity at a catalytic centre of that subunit but also by cooperation might be transmitted to the monomeric neighbours of the subunit. Such interactions lead to a multiplicity of conformation states, depending on the interacting ligands. A typical example is the allosteric property of pyruvate kinase from yeast. The enzyme reacts allosterically to the addition of phosphoenolpyruvate, ATP and ADP as well as fructose 1,6-diphosphate. Fructose 1,6-diphosphate transforms the sigmoidal saturation curve of the enzyme for phosphoenolpyruvate into a hyperbolic form (Hess & Sossinka 1974).

The kinetics of the enzyme can be described by the model of Monod *et al.*

if the model is extended to allow for hybrid states (Johannes & Hess 1973). Fig. 3 shows the computer output of the saturation function for a selected number of experimental curves with limiting curves for the R state and T state according to the model. The parameters in Table 2 demonstrate that the enzyme operates with an allosteric constant, L_0, of 60 000 and a non-exclusive binding coefficient, c, of 4×10^{-3} (on the basis of four monomers), which can be detected by physical and chemical means.

TABLE 2

Parameters in the hybrid model of pyruvate kinase from *S. carlsbergensis* (Johannes & Hess 1973).

Ligand	K_R/*mol* l^{-1}	K_T/*mol* l^{-1}
Phosphoenolpyruvate	1.9×10^{-4}	4.8×10^{-2}
Fructose 1,6-diphosphate	2.0×10^{-4}	$> 2 \times 10^{-2}$
ATP	$> 1 \times 10^{-1}$	9.3×10^{-3}

The model assumes four monomers. The allosteric constant, L_0, is 60 000, the non-exclusive binding coefficient, c, is 4×10^{-3} and q is 6.6.

Fig. 4 is the reaction scheme revealed by a detailed analysis of the model. The least consideration is that the kinetics can be attributed to two symmetric states as well as a hybrid state. Table 3 summarizes the thermodynamic parameters which result from the values of the various equilibrium steps. Although the hybrid state is formed from either non-hybrid state with a free energy change of 3.1 or 3.4 kcal/mol, I should mention that the formation of the hybrid is favoured by 1.1 kcal/mol enzyme over the formation of non-hybrid states.

TABLE 3

Thermodynamic parameters derived from the values of the various equilibrium steps (see Fig. 4) of the model of pyruvate kinase (Johannes & Hess 1973): pH 7.0 and T 25 °C.

Constants	$-\Delta G°$/*kcal mol*$^{-1}$
$L_0 = 60\,000$	6.5
$qH_0/2 = 315$	3.4
$2H_0 = 191$	3.1

In steady-state conditions the deviation from equilibrium of the reaction catalysed by pyruvate kinase in glycolytic systems as indicated by ΔG is about −5.0 kcal/mol, a value which compares well with the magnitude of the allosteric constants of the tetrameric enzyme with its four binding sides. However, it is at present impossible to relate these values to a detailed balance of the ligand

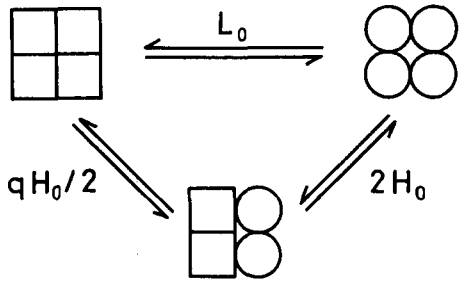

FIG. 4. Schematic representation of the kinetic model of yeast pyruvate kinase with the equilibrium constants for the steps shown (Johannes & Hess 1973).

binding involved in the steady state of the enzyme conformation; hardly any serving theoretical basis is available to us (Weber 1972). Also, one of the controlling ligands, fructose 1,6-diphosphate, is not stoichiometric participant in the reaction catalysed by the enzyme; its concentration and, therefore, its chemical potential as a ligand of pyruvate kinase, is determined by the activity of other components of glycolysis, such as phosphofructokinase or fructose-1,6-diphosphate phosphatase.

In general, this analysis illustrates that for a shift of the equilibrium from the T state to the most active R state a free energy change of 6.5 kcal/mol enzyme (at most) must be paid. This is the price for control of the activity of the enzyme and, therefore, the glycolytic flux in the lower pathway. At present, it is difficult to relate quantitatively the free energy changes of the enzyme to the glycolytic flux because of the lack of reliable data for *in vivo* conditions. It is certainly useful to remember that with a molarity of tetrameric pyruvate kinase in yeast of 20 μmol/l a permanent shift from the T to the R state would need a ΔG of 0.13 cal/mol. This is about 1–2% of that which is steadily available in terms of ATP (ΔG –7.3 kcal/mol), being present in a concentration of roughly 1 mmol/l, and should be compared with a glycolytic flux of ethanol of 30 mmol l^{-1} min^{-1}, which generates a rate of change of free energy of 220 cal/min.

The use of energy for the control of electron transfer along the respiratory pathway of mitochondria can well be demonstrated for cytochrome b_T. The redox state of this enzyme depends strongly on the rate of electron transfer, and a change of its redox potential over a span of almost 250 mV has been observed (Wilson *et al.* 1973). An analysis of the state of various cytochrome components in intact yeast during conditions of rapid electron flux as well as a minimum electron flux clearly illustrates that cytochrome b_T may be either

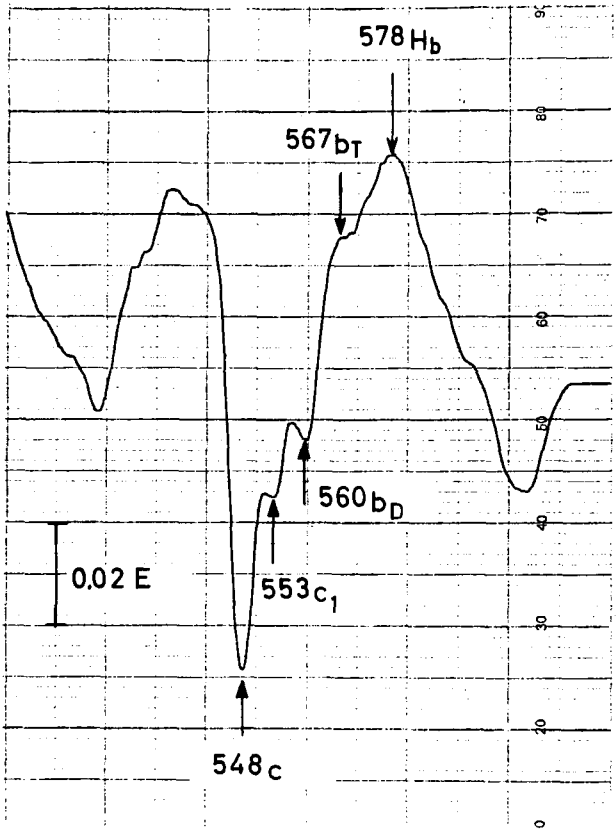

FIG. 5. Low-temperature difference spectrum (anaerobic minus aerobic) of a suspension of yeast cells. An upward deflection indicates increased reduction of the cytochrome components. The component with a maximum at 578 nm is yeast haemoglobin (Hb) but all other components are cytochromes: low electron flux.

more reduced or oxidized compared to the reference carrier cytochrome *c* (B. Hess & H. Kuschmitz, unpublished results). A comparison of the low flux state (Fig. 5) with the high flux state (Fig. 6) shows that at low flux cytochrome b_T is reduced whereas cytochrome *c* is oxidized. The reverse (Fig. 6) is found when a high flux is imposed: cytochrome *c* is reduced while cytochrome b_T is oxidized. This indicates that the redox potential of cytochrome b_T in a high flux changes to become equipotential with that of cytochrome *c*. Thus, depending on the rate of flux along the electron transfer pathway, the redox state of cytochrome b_T may vary over a large range, thereby indicating the conformational change of this cytochrome. This depends on the state of

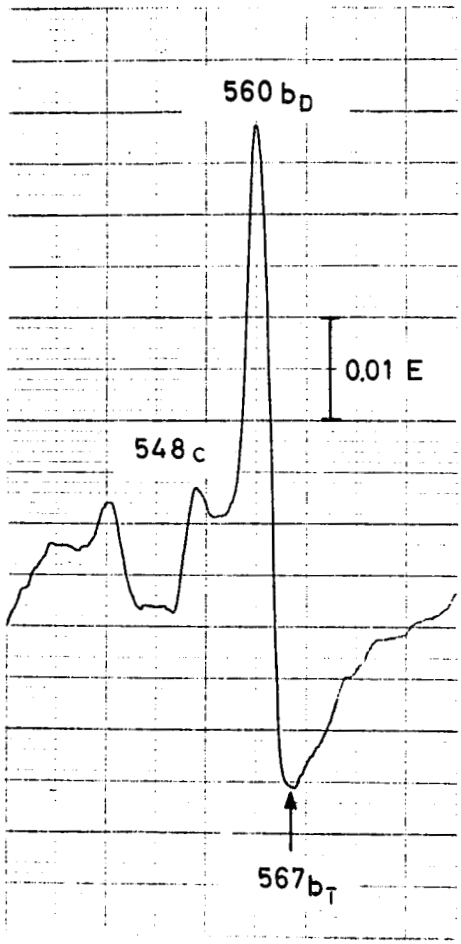

FIG. 6. Low-temperature difference spectrum (oxygen and ethanol minus oxygen) of a suspension of yeast cells. An upward deflection points to increased reduction of the components (cytochromes *c*, b_D and b_T) shown; high electron flux.

energization, as represented schematically in Fig. 7 (B. Hess & H. Kuschmitz, unpublished results). Whereas the mechanism of this observation has not yet been detailed, its general significance for the problem of energy use for control of electron pathway is obvious.

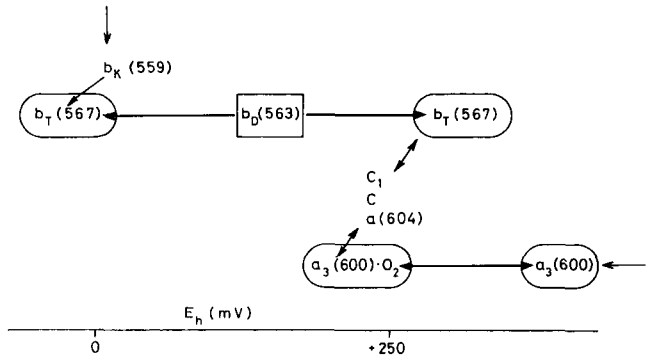

FIG. 7. Schematic representation of the interaction of cytochromes in yeast cells: arrows illustrate the pathways of electron transfer; scale gives an approximation of the redox potential relative to cytochrome *c*.

MICHAELIS AND NON-MICHAELIS ENZYMES

The essential difference between the performance of near-equilibrium enzymes with Michaelis kinetics and the regulatory enzymes with sigmoidal kinetics lies in the fact that in the latter the controlling signals, displayed as changes in the concentration of ligands, result in flux changes by amplification. Given a tetrameric enzyme with an allosteric constant, L_0, of 10^4, the reaction velocity is raised from 10 to 90% of V_{max} by a concentration change (Δc) of only 5% compared to that which is needed to achieve the same flux increase in a Michaelis enzyme. This property has important kinetic implications with respect to the response time of allosteric enzymes to controlling perturbations, such as those in hysteretic phenomena. With respect to the energy requirements, the thermodynamic consequences are drastic, namely enabling a small Δc (and corresponding ΔG) to contribute significantly to the control of the system's flux. Thus, it is the built-in structure of oligomeric enzymes that allows a flexible and 'cheap' control of turnover, which Michaelis enzymes cannot do.

STABLE AND UNSTABLE STATES

The regulatory enzymes allow the maintenance of a variety of dynamic states, all resulting from the use of energy for enzymic control. A typical example lies in the various transitions of glycolysis in yeast and other species observed on perturbation of the reaction pathway by changes in parameters such as the rate of addition of glucose, the ratio of ATP/ADP (Hess 1968) or

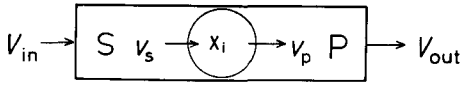

FIG. 8. Model describing the kinetics of phosphofructokinase of yeast. Substrate is fed into the substrate pool (S) at a rate V_{in} where it is turned over (at v_s) into product (P) by a mechanism X_i.

$$v_s(S,P) = v_p(S,P) = V(S,P)$$
$$\frac{d[S]}{dt} = V_{in} - V(S,P)$$
$$\frac{d[P]}{dt} = V(S,P) - V_{out}$$
$$\alpha = [S]/K_s$$
$$\gamma = [P]/K_p$$

the adenylate energy charge (see Chapman *et al.* 1971). Depending on the state of operation during non-equilibrium conditions when a net flux is maintained monotonic transitions, overshoots or oscillations may be produced.

An important regulatory enzyme responsible for the complex dynamics of glycolysis is the allosteric phosphofructokinase. The regulatory function of the enzyme can be described by a saturation function on the basis of the Monod–Wyman–Changeux model. We call the model (Fig. 8) an 'enzyme-machine'. The machine is fed with the substrate at a rate of V_{in} to supply a substrate pool S. The substrate, with a rate of v_S, is turned over into the product by a reaction mechanism X_i, which represents the allosteric phosphofructokinase. Finally, the product disappears in a sink (at a rate V_{out}).

The homogeneous system (neglecting diffusion) is described as a quasi-steady-state approximation (Goldbeter & Lefever 1972; Th. Plesser, V. Schwarzmann & B. Hess, unpublished results) by equations (5) and (6),

$$\dot{\alpha} = \frac{V_{max}}{K_{R(S)}}(V_{in} - V[\alpha, \gamma]) \tag{5}$$

$$\dot{\gamma} = \frac{V_{max}}{K_{R(P)}}(V[\alpha, \gamma] - V_{out}) \tag{6}$$

where the reaction velocity, $V(\alpha, \gamma, \delta)$,—on the basis of the Monod theory—is given by (7):—

$$V(\alpha,\gamma,\delta) = \frac{k\alpha(1+\alpha)^{n-1} + lc\alpha L'(1+c\alpha)^{n-1}}{(1+\alpha)^n + L'(1+c\alpha)^n} \tag{7}$$

$$L' = L_o\left(\frac{1+g\gamma}{1+\gamma} \cdot \frac{1+\delta}{1+h\delta}\right)^n$$

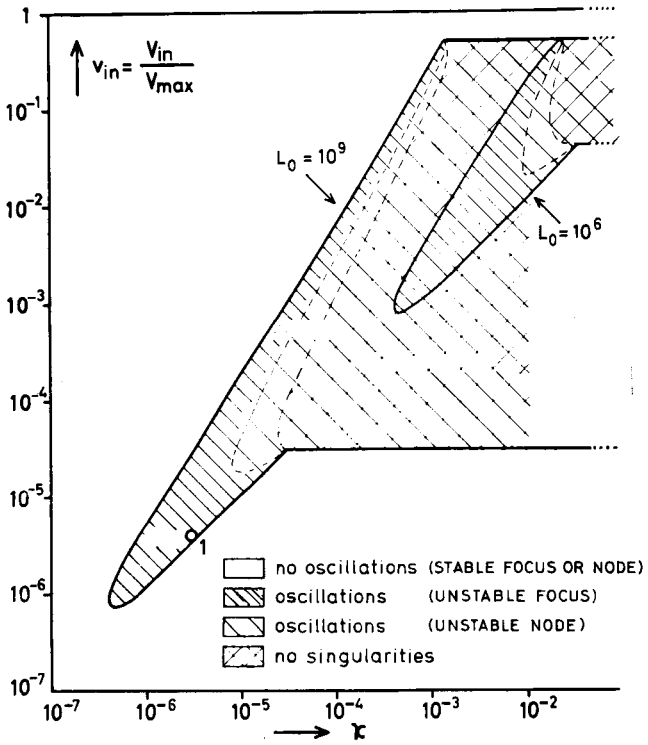

FIG. 9. Relationship between the rate of substrate input ($v_{in} = V_{in}/V_{max}$) and the sink constant ($\varkappa$) of phosphofructokinase in yeast for two different allosteric constants ($L_0 = 10^9$ and $L_0 = 10^6$).

and $L_o = T_o/R_o$. Furthermore, $\alpha = [S]/K_{R(S)}$, $\gamma = [\text{Activator}]/K_{R(A)}$, $\delta = [\text{Inhibitor}]/K_{R(I)}$, $c = K_{R(S)}/K_{T(S)}$, $g = K_{R(A)}/K_{T(A)}$ and $h = K_{R(A)}/K_{T(I)}$. Here, $K_{R(S)}$ and $K_{T(S)}$ are Michaelis constants for the R and T state, respectively.

An analysis of the dynamic non-equilibrium states of the enzyme model (Fig. 9) illustrates the variation in the rate of substrate input as a function of the sink constant. For sink constants between 10^{-6} and 10^{-5}, an increase in the rate of substrate addition (parallel to the ordinate through point 1) first leads through a domain in which a stable node or focus and a steady-state turnover are observed with monotonic transitions or overshoot phenomena, respectively. Then, a domain is passed in which oscillations are observed until finally a stable domain is reached.

Typical states can be illustrated graphically with a computed phase plan relating the concentration of substrates to the concentration of the products at any given time. Fig. 10 shows the response of the enzyme to a perturbation in

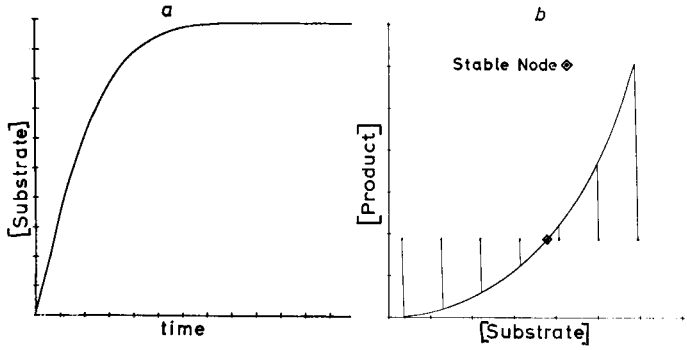

FIG. 10. Computer representation of the solutions of the equations given in Fig. 8 with $L_0 = 10^9$ and $c = 10^{-3}$: (*a*) monotonic transition to a steady state; (*b*) concentration ratios—the system passes through to a stable node irrespective of the initial conditions.

the stable domain: Fig. 10*a* shows the monotonic transition to a steady state for the substrate concentration (plotted against time for given conditions). In Fig. 10*b* the phase plan shows that from any concentration ratio at zero time the system passes through to a stable node at which the concentrations of substrates and products, in steady-state conditions, are invariant.

In many metabolic pathways, an overshoot phenomenon is observed. This overshoot is the expression of a transient unstable turnover after a perturbation. Fig. 11*a* demonstrates that the concentration of the substrate characteristically changes with time until a stable focus is reached and (Fig. 11*b*) from any initial concentration of substrate and product the stable focus will be reached and maintained as long as the conditions hold.

The third case is illustrated in Fig. 12. Fig. 12*a* shows the periodic change of concentration of substrate over the time. The phase plan (Fig. 12*b*) shows that, with a given input rate, the enzyme operates in the oscillating domain and the concentrations of substrate and product at any given initial concentration ratio inside or outside approach the limit cycle.

Glycolytic oscillations occur over a large range of input conditions and can be observed in intact cells as shown in Fig. 13 in which a cut-out of 65 cycles with a period of about 19 s is recorded with an overshoot transient until the system settles in the oscillatory domain (Boiteux & Hess 1968). The limit cycle of the model given above fits the experimental oscillations with respect to the oscillatory range of the substrate injection rates, the enzyme activities, the period and amplitude as well as the phase shift responses. Also, the ability to synchronize to external periodic action, to generate subharmonics of the driving frequency,

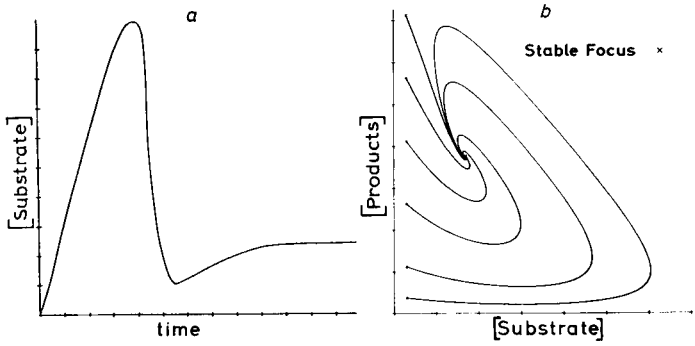

FIG. 11. Computer representation of the solutions of the equations given in Fig. 8 with $L_0 = 10^9$ and $c = 10^{-2}$: (*a*) variation in substrate concentration with time; (*b*) despite several initial conditions, a stable focus is reached.

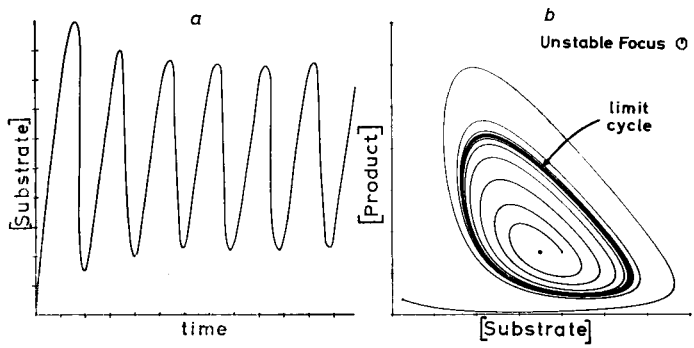

FIG. 12. Computer representation of the solution of the equations given in Fig. 8 with $L_0 = 10^9$ and $c = 10^{-2}$, (*a*) periodic change in concentration of substrate with time; (*b*) unstable focus: concentrations approach the limit cycle.

and to react to stochastic signal inputs can be demonstrated (Hess *et al.* 1969; A. Goldbeter, A. Boiteux & B. Hess, unpublished results).

The general significance of glycolytic oscillations cannot yet be evaluated. However, a periodic change in glycolytic activity might have a significant effect on the adenylate energy charge, as defined by Chapman *et al.* (1971). An analysis of the adenylate energy charge in conditions of an unstable steady state and of a limit cycle points to a possible function of metabolic oscillations. Oscillations could give rise to an alternating operation of metabolic pathways with ATP-utilizing functions being favoured for short time intervals before periodic drops in the energy charge in which temporarily energy-yielding proces-

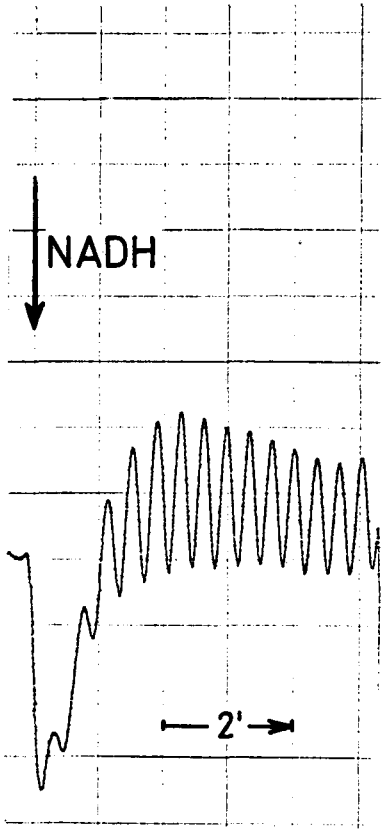

FIG. 13. Fluorimetric record of reduced pyridine nucleotide (NADH) during oscillation of glycolysis in intact yeast cells (increase of NADH concentration is indicated by a downward deflection). The glucose injection rate is 220 mmol l^{-1} h^{-1} (Boiteux & Hess 1968). A time interval of two minutes is indicated.

ses like glycolysis are activated to regenerate ATP (Goldbeter 1974). The difference between the affinities for hydrolysis of ATP on the limit cycle and at the steady state is generally +2 kcal/mol. Thus, the energy utilization for control of a regulatory enzyme in the oscillatory domain might be an energetic advantage for the overall metabolic functions, compared to a steady-state operation.

So far, I have discussed the consequences of energy utilization for control of regulatory enzymes for the homogeneous case in which diffusion is neglected. It seems to me appropriate to discuss here the inhomogeneous case, in which chemical waves and concentration gradients of cyclic AMP are generated

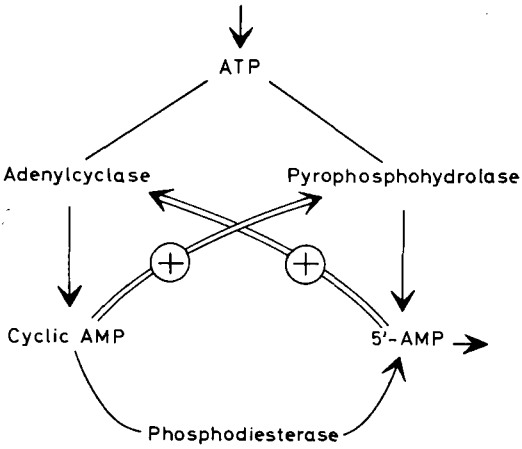

FIG. 14. Schematic representation of the adenylate cyclase system in the cellular membrane of *D. discoideum* (Gerisch *et al.* 1974).

extracellularly as a means of communicating chemical information between cells of the slime mould *Dictyostelium discoideum*, resulting in the triggering of chemotactic movement towards the incoming chemical signals. This process finally leads to cell aggregation and the formation of multicellular masses, which then differentiate. This system exemplifies the self-organization of spatial patterns by chemical communications starting with a layer of randomly distributed identical cells.

The aggregation territories are controlled by centres which release the chemical pulses of cyclic AMP with a frequency of 12–18 Hz. The pulses are propagated from cell to cell as excitation waves which, in a uniform cellular layer, spread with a constant speed of 40–50 μm/min depending on the cellular density. The waves are either concentric or spiral. Formally, the system can be treated as a set of diffusion-coupled oscillators, in which the *D. discoideum* cells operate by receiving, amplifying and ejecting periodically cyclic AMP signals in synchrony with cellular motion and oscillation by cytochrome *b* (Hess *et al.* 1975; Gerisch & Hess 1974; Gerisch *et al.* 1974). The response of the cells to cyclic AMP can be demonstrated by optical techniques.

One critical function in this process is the mechanism of generation of cyclic AMP pulses, which is illustrated in A. Goldbeter's model (unpublished work), based on the kinetic data of Rossomando & Sussman (1973). Here, the adenylate cyclase (see Fig. 14) is the regulating allosteric enzyme, which in given conditions and suitable activation by 5'-AMP readily settles into a limit cycle around a non-equilibrium unstable stationary state with a unique period in-

dependent of initial conditions. The model describes well the experimental observations. Again, the allosteric constant of 10^6 of the model points to the energy which must be used in control of the generation of propagating waves of cyclic AMP serving as a spatial chemical orientation pattern for morphogenesis. This is a new enzymic system for the transmission of information between cells—acceptably comparable to the acetylcholine-receptor–cholinesterase system (by which nerve pulses between neural cells are transferred over the synaptic space).

CONCLUSION

Energy is dissipated in the maintenance of non-equilibrium states (as defined by Glansdorff & Prigogine 1971) and, thus, also serves for control of the activity of enzymes, which are capable of responding to controlling chemical signals through their oligomeric and cooperative structure. This energy for control leads to complex dynamic patterns which are the essential features of the spatial temporal organization of living systems (Hess & Boiteux 1971). Analysis of the dynamic states reveals that stability is not ensured if far-off equilibrium conditions in metabolic systems controlled by non-linear rate laws are maintained. More than one steady state can be observed, rotations around a steady state might be sustained, following a limit cycle behaviour and finally when coupling to diffusion becomes significant spatial propagation of the system may be induced. The source of these phenomena are the classical key steps of multienzyme systems, where the strong non-linearity is produced. In the context of this presentation only a few cases have been discussed in which the energy requirement for control functions can be specified. The requirements can be demonstrated for the control of soluble as well as membrane-bound enzymes and receptor protein functions, such as pyruvate kinase, phosphofructokinase, adenylate cyclase or cytochromes. It is tempting to speculate how the energy cost for control rises with the complexity of the metabolic units to be controlled, as in the case of the coordination of various pathways. The occurrence of futile cycles with several allosteric enzymes involved at the same time might easily increase the cost of energy up to 10%—as has been experimentally tested—with the advantage of gaining a much more precise way of controlling cellular processes (Newsholme & Start 1973; Clark *et al.* 1974).

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Discussion

Kornberg: The energy cost of futile cycles can be fatal. Human patients, and animals, with the genetic defect of malignant hyperthermia are unusually sensitive to the anaesthetic halothane; if subjected to it, their body temperature rises catastrophically and they may die on the operating table. Hird and his colleagues have shown that this phenomenon is accompanied by an efflux of calcium ions from the sarcotubular system into the myoplasm, where the ions stimulate both glycogen breakdown and muscle contraction: the muscle, in effect, becomes an ATPase converting glycogen into lactic acid on a massive scale (Denborough *et al.* 1973). Work in Lardy's laboratory has further shown that, in affected pigs, the heat production induced by halothane is directly related to an accelerated rate of fructose 6-phosphate cycling (Clark *et al.* 1973), so that both the reactions (1) and (2) occur simultaneously.

$$\text{fructose 6-phosphate} + \text{ATP} \rightarrow \text{fructose 1,6-diphosphate} + \text{ADP} \quad (1)$$

$$\text{fructose 1,6-diphosphate} + \text{H}_2\text{O} \rightarrow \text{fructose 6-phosphate} + \text{P}_\text{i} \quad (2)$$

The net sum of these reactions is, of course, $\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{P}_\text{i}$. This could be the ATPase observed by Hird and his colleagues.

Hess: Clinical pathology offers several instances in which futile cycles on different levels of organization might lead to a fatal loss of energy. A classical example is thyrotoxicosis. There is also an interesting case of hypermetabolism—clinically similar to thyrotoxicosis—which is due to a development of giant mitochondria in the muscle of the patients. These mitochondria display an extremely active ATPase activity, which explains the uncoupled oxidative phosphorylation (Luft *et al.* 1962).

McClare: Is the oscillation in the slime mould related to the oscillation in cytoplasmic streaming observed in the fused state? I recall a time-lapse film which vividly showed pulsations due to the inward and outward streaming movements.

Hess: We observed changes of light scattering, which indicate pseudopodial activity, cell elongation, and increased cell-to-cell adhesion. Oscillations as well as the cyclic AMP response of light scattering might be due to changes of any of these functions. Indeed, recently it has been shown that pseudopod extensions can be triggered by cyclic AMP.

Lipmann: What does cyclic AMP do to the cell? Is the triggered reaction a phosphorylation of a protein?

Hess: Cyclic AMP is bound to receptor sites located at the cellular membrane. Titration experiments with 1nM-cyclic AMP showed that a molecule/cell ratio of 3×10^3 still leads to detectable responses of the light scattering signal. This indicates a limited number of binding sites at the cellular surface. The dissociation constant of the cyclic AMP receptor complex is in the order of 0.1–0.2 μmol/l or lower. Incubation of cells which are competent to aggregate demonstrates a binding of cyclic AMP five seconds after nucleotide addition which decreases almost completely within five minutes, proportionately with the appearance of 5'-AMP in the intercellular space. In this type of experiment, excessive cyclic GMP is present to inhibit phosphodiesterase to a high degree. In the absence of the inhibitor 95% of added cyclic AMP is hydrolysed by the cell-bound phosphodiesterase within a five second period and, therefore, no binding can be detected later on. There must be a highly specific mechanism by which the receptor proteins transfer to other cellular functions (such as pseudopod acitivity, periodic synthesis and release of new cyclic AMP, etc.) the information that it carries cyclic AMP or not. Maybe, this information is passed by means of the phosphorylation of the protein. We have suggested that the cyclic AMP receptor might well function as a protein kinase.

Lipmann: Do the cells really move?

Hess: Yes, they do. We can demonstrate this directly on film. Within an aggregation territory, chemotactic material, such as cyclic AMP, is released from the aggregation centre in pulses with periods of 2.5 to 5 min. The pulses of the chemotactic activity are propagated as an excitation wave from inside the territory to outside with a constant speed of between 40 and 500 μm/min depending on the cellular density in the territory. The cells around a centre respond with orientated cell movement of the same frequency.

Huxley: These oscillations resemble those that can occur in excitable membranes, where positive feedback through the change in membrane potential increases the permeability to sodium thereby increasing the change in membrane

potential further. In ordinary Ringer's solution, we observe a single spike rather like the overshoot in Fig. 11, but when we lower calcium concentration the oscillation is maintained, or a few cycles of oscillation occur in response to a single stimulus. The analogy is close because the equations for spread by diffusion are identical to the equations for the spread of charge along a resistive and capacitive structure such as a nerve fibre.

Hess: The phenomenon of intercellular communication and periodic aggregation of *Dictyostelium discoideum* controlled by cyclic AMP can be treated formally as a system of diffusion-coupled oscillators and we have pointed to its analogy with the system responsible for the transmission of nerve impulses through a synaptic cleft. The elements of the slime mould system resemble the acetylcholine-receptor-cholinesterase system where also a binding protein is associated with a hydrolase. A chemical analogue of the slime-mould system or even the neural system is the oscillatory oxidation of malonic acid in the presence of bromine, which is commonly called the Belousov–Zhabotinsky reaction. In all cases periodic activities are coupled to diffusion leading to the production of chemical waves. They resemble those

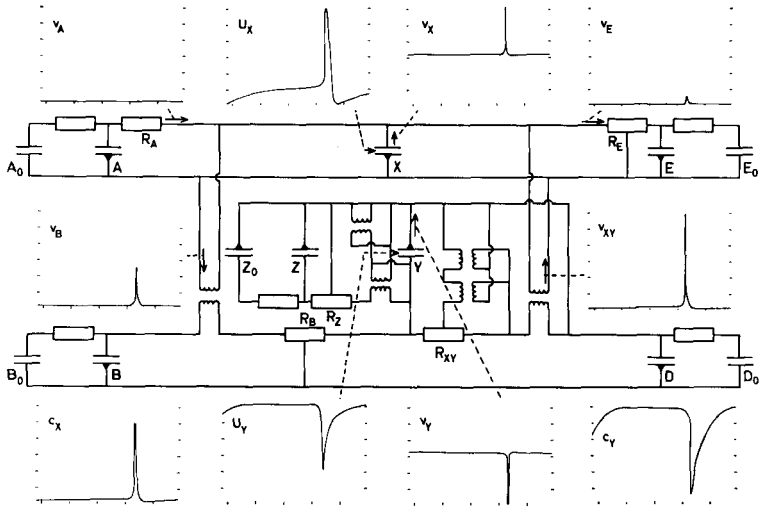

FIG. 1 (Hess). Network of a chemical reaction system: A, B, D, E, X, Y and Z are chemical compounds corresponding to energy storing capacitors. A_0, B_0, D_0, E_0 and Z_0 are chemical compounds serving as reservoirs to maintain constant concentrations of A, B, C, D, E and Z, respectively. The oscillation proceeds by charging capacitor Y through R_B up to a threshold value against discharge through R_Z. Then, reaction R_{XY} fires and discharges the capacitor Y: v is the rate of reaction c_X and c_Y designate the variations in the concentration of X and Y; U_X and U_Y are the normalized chemical potentials. The full time scale is 52 units. (From Busse & Hess 1973 with permission).

occurring in the excitable membrane. In the chemical system we have studied the property of information transmission (Busse & Hess 1973). Analysis of the kinetic laws of the elementary chemical system demonstrates instability and periodicities in time and space as well as localized structures. From the chemical reaction scheme a network can be derived as in Fig. 1, where the time dependence of some variables in extreme non-equilibrium conditions is illustrated. A concentration (charge) is accumulated up to a threshold value; subsequently, the capacitor (Y) is discharged and a new process starts. The system of difference and differential equations can be solved by computer and demonstrates how chemical pulses are propagated and, furthermore, how an imposed short signal is propagated through the system. The results obtained in the network analysis have been demonstrated experimentally. The network treatment of the chemical system demonstrates the close analogy between the various systems discussed.

Wilkie: How is this thermodynamic treatment of chemical processes related to, for example, the treatment by Prigogine (1955), in which the stress is on rates of entropy creation?

Hess: In the classical relation by De Donder (1934) the rate of entropy production is equated with the temperature (T), the affinities (A_i) and the velocities (v_i) of a reaction system with a number of components, i (equation 1).

$$\frac{\mathrm{d}S}{\mathrm{d}t} = T^{-1} \sum_i A_i v_i > 0 \tag{1}$$

It should be interesting to analyse quantitatively the entropy term with respect to control. However, I feel that we need much more reliable data for *in vivo* conditions at various temperatures to obtain reasonable results.

Huxley: Is this assuming some linearity in the system?

Hess: The results obtained so far for some glycolytic enzymes, such as yeast glucosephosphate isomerase (Hess *et al.* 1972), muscle enolase (Bücher & Siess 1969) or lactate dehydrogenase or glycerolphosphate dehydrogenase (Bücher & Rüssmann 1963), justify the assumption of nearly linear conditions.

Wilkie: I admit, this is a much better way than by consideration of entropy creation.

Gutfreund: Is cytochrome *b* an allosteric enzyme? Is it a monomer?

Hess: Recently, it has been shown that cytochrome *b* isolated from yeast mitochondria is definitely a dimer, on the basis of chemical analysis. Stop-flow experiments by Dr Weiss in my laboratory support this result kinetically.

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General Discussion

ADENOSINE PHOSPHATES AND ADENYLATE KINASE

Wilkie: Dr Cohn, you said that adenylate kinase is an important factor in tissues in which biosynthesis is proceeding because it is the only agent which can reconvert AMP into ATP, but in muscle the situation is reversed: adenylate kinase is only ever used as a last resort; the AMP produced in consequence is removed by deamination, so the adenyl part is thrown away. All muscles contain these deaminating enzymes. Do other tissues do the same?

Cohn: Cells can synthesize AMP, but how is it phosphorylated to ATP or to ADP, the substrate in oxidative phosphorylation for ATP? It must be recycled. The only way that the monophosphate can be converted into the diphosphate is by adenylate kinase. M. Glaser & P. R. Vagelos (unpublished work, 1974) have prepared a temperature-sensitive mutant of *E. coli* lacking adenylate kinase that can synthesize neither protein nor nucleic acid.

E. Krebs: One commonly sees the statement that the concentration of each of the three adenosine phosphates does not vary during muscle contraction, but I wonder whether we really know what happens to the amounts of different nucleotides *in vivo*.

Wilkie: But we do. Hultman (1967) and others take muscle biopsies of people performing exercise. The concentration of ATP stays constant until the person is on the point of exhaustion, whereupon it drops; a really motivated athlete can go on until his ATP has dropped to about 50% of its normal value at which point he is about to go into rigor mortis!

E. Krebs: Then metabolic regulation as a result of changes in the concentration of ATP relative to the concentrations of AMP and inorganic phosphate (the Atkinson energy charge consideration) presumably has no place in the regulation of skeletal muscle metabolism in contraction because the ATP concentration is always constant.

Wilkie: However, the amount of ADP changes a lot, proportionately. Good

evidence indicates that the concentration of ADP, among other things, regulates oxidative phosphorylation—I imagine in humans as well as in frogs.

Glynn: If the muscle contains an AMP deaminase and adenylate kinase, why isn't all the ATP continually leached out of the system?

Wilkie: I suppose that little AMP is produced in the course of normal contraction. Certainly, little of the deaminated form, IMP, is seen.

Cohn: When do you find AMP?

Wilkie: It is found during extreme exhaustion and also if creatine kinase is poisoned with fluorodinitrobenzene, which effectively lowers the ATP concentration so raising that of ADP, AMP and IMP (Dydyńska & Wilkie 1966; Curtin & Woledge 1975).

Cohn: ADP is then hydrolysed. IMP cannot substitute for AMP in the adenylate kinase reaction.

Kornberg: I'm getting confused, because we seem to be talking about three different things. In general, I agree with Dr Krebs and Professor Wilkie that the accumulation of AMP in a muscle is a cry of distress—the body is then running out of everything. It is also true, as Dr Cohn emphasized, that the biosynthetic reactions in the cell (we're not now talking about muscular contraction, i.e. mechanical work, but about biosynthesis, the chemical activation and combination of amino acids and of fatty acids) always yield AMP and not ADP. Therefore, the adenylate kinase reaction is essential if that AMP is to be reused. Thirdly, Dr Krebs introduced, but possibly this is a red herring, Atkinson's concept of energy charge. Atkinson put up the interesting idea that the ratio $([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP])$ in a cell determines whether some regulatory enzymes that are susceptible to this type of control are either speeded up or inhibited by alterations in this ratio. I find it difficult to evaluate how physiologically significant this is, because if one plots the rate of many enzymic reactions that are said to be so controlled against the energy charge, one usually finds that no major effect is observed up to an energy charge of about 0.8, beyond which the slope of the rate plot drops steeply. Even over a wide range of physiological stresses, one finds that, in general, the ratio of $([ATP] + \frac{1}{2}[ADP])$ to the total nucleotide pool does not change much, and that the energy charge seems always to be set not very far from 0.8. How significant is an apparently invariant ratio?

Cohn: I don't know why Atkinson chose that particular ratio. At equilibrium, as far as adenylate kinase is concerned, we should consider the ratio $[ADP]^2/[ATP][AMP]$. Also, I don't know why he calls that an energy charge.

Wilkie: I was contrasting the scenery in a synthetic cell with that in a muscle cell because, since the equilibrium constant for adenylate kinase is close to one,

AMP has to be removed before the reaction will produce much ATP. That, of course, is wasteful.

Klingenberg: Adenylate kinase and myokinase are localized completely differently in the two tissues; hepatic adenylate kinase is found in the perimitochondrial space attached only to the mitochondria. No other adenylate kinase exists in the liver. Myokinase in muscle, which is not the same enzyme as adenylate kinase, is located in the cytosol.

Lipmann: In measuring the synthesis of fatty acids or proteins in which AMP is formed, one should not use, as one usually does, phosphocreatine and creatine kinase to reconvert ADP into ATP, but rather myokinase and the other kinases. When this has been tried, the results have been much more satisfactory.

Woledge: I can see no real conflict between people who envisage a small change in nucleotide concentrations for control purposes and those who talk about large changes of energetic significance in the concentration of ADP and AMP. Much ADP can be found in muscle, but the amount of free nucleotide is low. I have referred to a lack of change in the total amount of ADP in muscle after stimulation; the free ADP could, however, change considerably without detection and could act strategically in metabolic control.

Huxley: The big changes in the ratio of free ATP to free ADP can be detected only with difficulty.

Hess: In glycolysis only phosphorylase and phosphofructokinase respond to a change in concentration of AMP. Such changes have a significant function in the regulation of both enzymes and the overall glycolytic flux. For phosphofructokinase, changes in the concentration of ADP or ATP affect the activity in the physiological range to a small degree. However, the additional activating effect of an increase in concentration of AMP produces a drastic acceleration in the activity of phosphofructokinase in yeast up to 500-fold. In this control mechanism, adenylate kinase plays a significant amplifying role. Through the catalytic function of this enzyme a drop in the concentration of ATP and the corresponding increase of the concentration of ADP lead to an amplified increase in the amount of AMP. Thus, by the action of this enzyme, small changes in the concentration of ADP or ATP become effective through the action of an additional controlling ligand, which is enzymically equilibrated with both of them.

Cohn: Phosphorylase responds, too!

Roseman: In the biochemical studies discussed so far, nobody has mentioned tissue culture systems, which can beat synchronously. It seems to me that the problems with diffusion could be avoided with this system.

GEARING RATIOS IN ENERGY TRANSFORMATION

Huxley: Are there any cases known where the gear ratio of some energy transformation is variable? Dr Glynn intimated that in one situation no evidence could be found to suggest that this happened. When the potential difference against which a system has to work is altered, is there ever any provision for altering the stoichiometry so that, perhaps, the energy from more than one molecule of ATP could be utilized in overcoming the energy barrier? (If there is a small potential difference the system runs more freely.) In some ways this situation resembles the sliding system in muscle: rapid movements can take place without much chemical change because potentially interacting sites can pass each other without interacting. This was a comforting feature of the sliding filament theory, because it provided an explanation for the known relations between rate of chemical change (or rate of energy liberation) and speed of shortening. Needham (1950) pointed out that such a provision was needed to explain the observation that the rate of energy liberation does not increase linearly with speed of shortening—it levels off as the speed increases. This levelling off can be explained by sites passing each other without having time to interact. That shows a variable ratio between chemical change and movement. Is there any case where the amount of ATP used per chemical change produced varies with the potential that has to be overcome?

Glynn: Yes. In red cells the stoichiometry between sodium movement and ATP is constant, but in the snail ganglion neuron (Kostyuk *et al.* 1972) and in squid axon (Mullins & Brinley 1969; Brinley & Mullins 1974) the ratio between active sodium efflux and active potassium influx can be changed; if the ratio between sodium and ATP is constant, the ratio between potassium and ATP must vary.

Huxley: Does it vary with the membrane potential against which it is working?

Glynn: Yes, according to Kostyuk *et al.* (1972): they hyperpolarized the membrane and found that the Na^+ and K^+ fluxes produced by the pump became more nearly equal.

Keynes: In the squid axon, changing the membrane potential does not make any difference to the rate of outward movement of sodium ions.

Huxley: Not much work is done in moving potassium. Sodium is shifted against a large electrochemical difference.

Glynn: Mullins & Brinley (1969) found that as the sodium concentration in a squid axon was reduced the ratio of active sodium efflux to active potassium influx approach 1:1.

Keynes: The same is true in frog muscle.

Wilkie: In principle, if a scheme such as that put forward by Dr Oesterhelt

can operate, in which an electrogenic pump in one place sets up a potential difference that drives a chemical process elsewhere, in this event we should not expect any fixed stoichiometry.

Huxley: In that instance, there is no way in which one can add the potentials. One can add the fluxes from different driving sources because they are in parallel across one membrane. Are any cases known where the driving elements are added in series? Professor Keynes showed that the units in the electric organ are straightforwardly in series. If some chemical change needed 50 V to drive it, one could do it in this kind of way. Is there anything analogous to this on the molecular scale?

Keynes: The evidence shows that in an organ like the kidney much of the osmotic pumping work is a transfer of an isotonic solution of sodium chloride from the proximal tubule back into the blood against a very small gradient. If one calculates the total transfer of sodium and the total oxygen consumption in the kidney, one arrives at a figure of about 20 sodium ions moved/molecule oxygen consumed. But the limit for a sodium pump operating across a single membrane—assuming one ATP/Na^+—corresponds to only six ions/molecule oxygen. That illustrates a gearing.

Huxley: But that is being achieved by relatively gross arrangements, not on a molecular basis.

McClare: Perhaps an illuminating way of asking Professor Huxley's question about slip between ATP hydrolysis, or formation, and its effects is to ask whether the process is *quantized.* For example, Dr Oesterhelt's purple protein proton pump was quantized: one photon produced one H^+. If so, the same kind of mechanism could be implicated: namely, a quantum mechanical one.

Klingenberg: One example of such a situation is oxidative phosphorylation, in which the P/O ratio is diminished because part of the energy there before the formation of the ATP is withdrawn by the active output of ATP. As a result, less ATP is formed but the higher free energy of each ATP molecule means that the total energy (the number of ATP molecules multiplied by the free energy) is the same.

Keynes: The excretory organ is an example of where you might search for such a situation. It cannot cause a concentration gradient greater than about 5×10^7 if the system is driven by ATP, so that difficulties arise if the concentration gradient is greater. Kidneys, even in extremely dehydrated animals, are probably incapable of doing that, but certain insect excretory organs can remove the water from the faeces to leave an amount which is less than the vapour pressure of saturated potassium chloride. In the kidney, the evidence seems to point to a countercurrent mechanism. The mechanism in the insect excretory organs is still unknown.

ATPase SYSTEMS

Keynes: Several times in our discussion we have touched on the problem of coupling between energy production and ion gradients and the reverse. It seems to me that we ought to think more about systems other than the Na^+,K^+-ATPase. As Dr Glynn explained, we know a fair amount about the molecular basis of that active transport system. We are apt to forget the several other active transport systems about which we know much less. For example, active transport systems exist for chloride ions and for protons in the gastric mucosa. These definitely operate separately and in parallel. One question that springs to mind is, what drives the chloride pump? (The chloride pump is rather ubiquitous; it is not merely located in the gastric mucosa.) Also, in all insect excretory organs there is an extremely active electrogenic potassium pump about whose energetics we know almost nothing. Is it an ATPase? Berridge & Maddrell (unpublished results) have tried to demonstrate ATPase activity in these organs but failed totally. Ellory, in my laboratory, also failed. It is categorically not an Na^+,K^+-ATPase and there is no evidence to indicate that it splits nucleotides at all, yet it is highly active. We badly need to find out how it is coupled to the energy supply. I have also endeavoured to find a chloride-activated ATPase in the gastric mucosa, but without any success.

Professor Klingenberg, if we are seeking analogies between the mitochondrial system and some of these other ones, may not Na^+,K^+-ATPase be the wrong model? Perhaps we ought rather to consider one of the other ATPase systems.

Klingenberg: The question about the ionic requirement can only be partially answered. The isolated ATPase is only fragmentary in the reactions, compared to the membrane-integrated ATPase which catalyses a vectorial reaction. The ATPase reaction is a short-cut reaction requiring, as far as I know, only magnesium. However, in sonicated particles, ATPase is on the outside and one can examine the ionic requirements directly. I know of no evidence supporting a requirement for alkali ions.

Keynes: I believe that a hydrogen carbonate-stimulated ATPase from the gastric mucosa has been described. Could a specific requirement for hydrogen carbonate have anything to do with mitochondrial ATPase?

Lipmann: In the gastric mucosa, the hydrogen ions are produced by carbonic anhydrase.

Glynn: Carbonic anhydrase produces hydrogen ions but some other mechanism must shift them across the membrane.

Weis-Fogh: With respect to the potassium pump, it is conspicuous that the mitochondrial membrane is almost in direct apposition to the plasma membrane through which the potassium is pumped.

Keynes: Also, electron micrographs show little balls on stalks rising from the plasma side of the membrane (Berridge & Gupta 1967) as in mitochondria. This may be universal for the insect potassium-pumping system.

Glynn: Is there a concomitant hydrogen movement?

Klingenberg: Some bacteria contain a sodium–proton or potassium–proton exchange system which would be directly coupled to an ATPase, so that the two systems may be coupled very closely. It is not a direct ATP-driven sodium pump but a proton sodium pump.

Keynes: I don't think there is a movement of protons in the insect system; only potassium is transported extremely fast.

STORAGE OF ENERGY

Huxley: Another aspect of energy transformation is the storage of energy, in the straightforward mechanical way, by stretching an elastic structure. One system in which this probably happens is formed by the cross-bridges of muscle. Direct experimental evidence shows the presence there of an elastic element. If the cross-bridges act in steps of about 10 nm, the achievement of overall movements of that size from the distances of a few hundredths of a nanometre that are traversed in a normal chemical change needs a system that allows multiplication of the range of movement. Simple lever action is one possibility; this was incorporated into Dr McClare's suggestions and it is the basis of H. E. Huxley's ideas. Alternatively, with an elastic element which allows movements over longer distances, the energy expended in moving to the new position could be converted into mechanical work through the spring. How might mechanical energy be stored in other systems for energy transformation, either on the molecular or the macroscopic scale?

Weis-Fogh: An elastomeric effect can be obtained in two different ways. So far, people have considered mainly one basic type of storage in elastomers, namely in a homogeneous network of kinetically free chains. When elastic energy is stored, the entropy of the backbone conformation is decreased correspondingly. In those conditions, the amount of heat given off by stretching corresponds to the stored energy. In some proteins, for instance in resilin from arthropods or in abductin from mussels, this property of an ideal rubber is obeyed. At one point it was thought that the elastomer in vertebrates, elastin, behaved in this simple way, but the properties of elastin are more intricate; the difference may be relevant to the cross-bridges and to the 'neck' in the myosin molecule. According to biochemical analyses, elastin is an extraordinarily hydrophobic protein: only about 5% of the amino acid residues carry polar side chains. Also, both the soluble break-down products of native

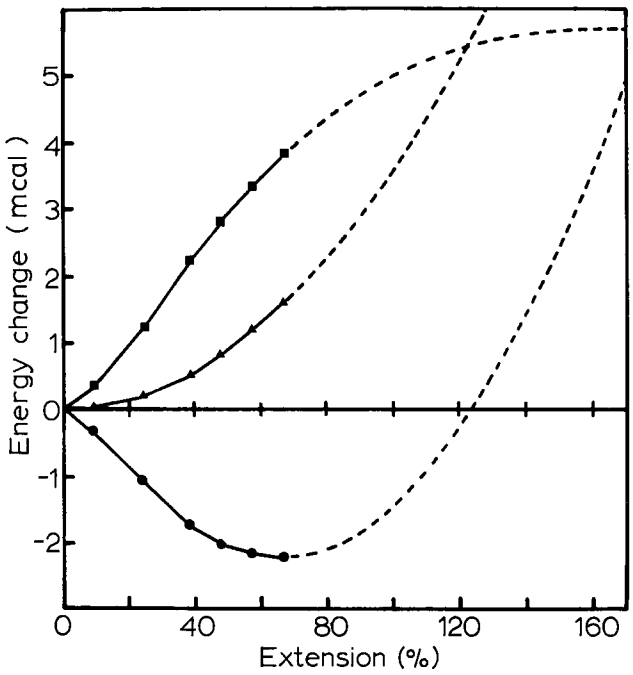

FIG. 1 (Weis-Fogh). An example of the change in work W (▲), free energy (■) and entropy (●) with extension of elastin in water at 24 °C. Up to 70% extension the calculated values are based on experimental data (full lines) (from J. M. Gosline, unpublished results).

elastin (α-elastin) and the soluble elastin monomer, tropoelastin (before cross-linking), will form reversible hydrophobic coacervates when the temperature is increased slightly (Smith & Carnes 1973). Water, therefore, appears to be a poor solvent for tropoelastin and a poor diluent for the cross-linked network. This means that the domain of a molecule dissolved in water will tend to be restricted compared with the domain occupied by the kinetically-free chain in a good solvent. If we imagine an assembly of such molecules linked together by cross-links in an aqueous environment, the resulting network will not be homogeneous but contain regions in which hydrophobic groups cluster. When the network is mechanically deformed, the strain not only alters the conformational entropy of the polypeptide backbones but there will be a tendency to increase the interface between dense hydrophobic regions and the free diluent, water, i.e. hydrophobic interaction becomes important. It is then feasible that work could be stored by the creation of new interfaces where the network is strained provided that the free-energy changes associated with this type of hydrophobic interaction are of the right sign and magnitude. Dr

J. M. Gosline and I have recently concluded an extensive series of calorimetric experiments (cf. Weis-Fogh & Andersen 1970) in order to test this hypothesis, using the thermodynamic data of Némethy & Scheraga (1962) as a basis for the calculations. An example is shown in Fig. 1 in which the work W done on the elastin sample by stretching it has been resolved into the free-energy change associated with hydrophobic interaction, ΔF_s, and the free-energy change, ΔF_{conf}, associated with the change in conformational entropy ($\Delta F_{conf} = -T\Delta S_{conf}$). In contrast to what one would expect from the simple kinetic theory, the entropy term of a slightly or moderately stretched network has become increased rather than decreased, up to about 70% extension (full lines). At higher extensions (broken lines) the usual entropy mechanism tends to dominate. These findings can be interpreted to mean that when elastin is stretched the dense hydrophobic regions become more accessible to water and increase their domains. Energy is then stored as a consequence of a restructuring of the water–protein interfaces.

From the point of view of the problems discussed here, this type of elastic storage of energy could work also for individual molecules or parts of protein chains and not only for a whole network.

CLOSING REMARKS

Huxley: During this meeting, we have not solved any of our problems, but we have all gained a new range of ideas to keep at the back of our minds when we brood about these inscrutable and complicated systems with which we experiment. In my own field—muscle—decidedly we must obtain a great deal more experimental information about the movements of the component parts of a muscle during a contraction before we can make direct use of what has been learnt about quantum events in other types of energy transformation. Although obviously extremely complex, the experiments should not prove insurmountably difficult. Many groups are studying muscular movement by X-ray diffraction and by attaching labels of various kinds to the molecules in order to get information about their mobility even during contraction at a fixed length. Such experiments reveal the existence of a whole level of complexity about which we know almost nothing and ignorance of which prevents any direct interpretation in terms of quantum mechanical events, which clearly will be the final explanation. Similar problems encumber the other systems we have been discussing, even though tremendous progress at a biochemical level has been charted for carriers in relation to active transport. However, the answer to the question of how splitting a bond in ATP produces the observed movement of the substrate still eludes us.

Finally, on behalf of all the members of the symposium, I congratulate Professor Lipmann on his 75th birthday.

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