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Reassessment of the Role of ATP *in Vivo*

BARBARA E. C. BANKS AND C. A. VERNON

Departments of Physiology and Chemistry,
*University College, Gower Street, London W.C.*1, *England*

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In 1941 Lipmann introduced the term "high energy phosphate bond". This concept has had profound effects on the development of biochemical thinking and has led to a considerable preoccupation with free energy changes, either actual or standard, associated with metabolic reactions and, in particular, with the hydrolysis of ATP. In this paper we wish to propose the view that the original concept put forward by Lipmann was ill-founded and that its effect is to divert attention from the genuine problem of the mechanism of events in which ATP takes part. We shall show that (a) the hydrolysis of ATP is a forbidden reaction in intermediary metabolism, (b) that the Lipmann concept would be appropriate for a closed system containing energy-linked reactions (of which there are no known examples in biochemistry) and (c), most importantly, that since real organisms are *open* and not *closed* systems even the direction of flow of matter through a particular unit step cannot be predicted from the associated standard free energy change but only from the properties of the whole system making up the steady state. In other words simple thermodynamic parameters are *irrelevant* in discussing whole organisms: these must be understood in kinetic and mechanistic terms. We shall illustrate these ideas by considering some metabolic processes and, in particular, we shall show that for oxidative phosphorylation the exclusion of simple thermodynamic considerations enormously simplifies the discussion and leads naturally to a plausible mechanism for ATP formation. We shall conclude by considering the factors which may have been important in the selection of ATP for its metabolic role. Although the values of the standard free energies of hydrolysis of phosphate esters are irrelevant *in vivo*, a critical account is given in the Appendix of the experimental data on which the accepted values are based. Brief accounts of these views have already been published. (Vernon, 1960; Banks, 1969, 1970.)

1. The High Energy Phosphate Bond Concept

The concept arose when it became evident that a particular nucleoside triphosphate (ATP) is unique in metabolism. In all organisms the degradation of primary foodstuffs (catabolism) is geared to the production of ATP which is then used to mediate a large number of synthetic reactions (anabolism).

Since catabolic processes are normally thought of as those processes proceeding with a decrease in free energy whereas, for the most part, the converse is true for anabolic processes, it has been supposed that one set of processes "drives" the other, and that ATP provides the essential link between them. For example, the anaerobic degradation of glucose can be represented by the stoichiometric equation

$$C_6H_{12}O_6 + 2\ ADP + 2\ H_3PO_4 = 2\ CH_3CH(OH)COOH + 2\ ATP + 2\ H_2O, \quad (1)$$

so that two moles of ATP are apparently formed for each mole of glucose degraded. On the other hand, the synthesis of urea can be represented by

$$2\ NH_3 + CO_2 + 3\ ATP + 2\ H_2O = (NH_2)_2CO + 2\ ADP + \\ + 2\ H_3PO_4 + AMP + H_4P_2O_7, \quad (2)$$

in which three moles of ATP are involved in the synthesis of every mole of urea.† Many metabolic processes can be represented by similar equations and the importance of ATP as a linking substance is obvious. It is, perhaps, worth pointing out that these stoichiometric equations are obtained by summing the unit steps making up the particular sequence thereby cancelling out the concentrations of all the intermediates. Consequently their relevance *in vivo* is by no means clear: we return to this later.

The essential point of Lipmann's concept was that ATP functions as a linking substance because it can store the energy released by degradative processes and can then use it, as required, to drive synthetic reactions. This idea arose because it appeared that the standard free energy of hydrolysis of ATP, and of certain other compounds with similar but subsidiary functions, had a considerably higher value (*c.* -10 kcal/mol) than that associated with most simple phosphate esters (*c.* -2 to -4 kcal/mol). In the event, as seen in the Appendix, this dichotomy of "high energy" and "low energy" phosphates proved to be less clear-cut than originally thought. However, this is not important to the argument. The reason for the selection of the standard free energy of hydrolysis as a measure of energy storing capability is that stoichiometric equations such as (1) and (2) can always be written as the sum of a set of equations one of which is the hydrolysis of ATP or its reverse.

In the generalized case of synthesis represented by equation (3),

$$XOH + YH + ATP = XY + ADP + H_3PO_4 \quad (3)$$

it is possible to write this as the sum of equations (4) and (5), i.e. the hydrolysis

† This is an oversimplification since one of the nitrogen atoms comes from aspartic acid. The reaction is discussed in detail later. The formation of AMP and inorganic pyrophosphate will be considered in a later section.

of ATP and the direct synthesis of XY.

$$ATP + H_2O = ADP + H_3PO_4 \quad (4)$$

$$XOH + YH = XY + H_2O \quad (5)$$

If ΔG_5° is positive, i.e. the direct synthesis of XY is unfavourable thermodynamically, then the biological synthesis can still proceed providing that ΔG_4° is large and negative. In other words, the free energy of hydrolysis of ATP "drives" the synthesis of XY.

It is now natural to introduce the idea of *efficiency*. The synthesis of XY requires only that ΔG_3° be negative: hence if ΔG_4° is numerically larger than ΔG_5° the difference is said to be "wasted" free energy. In other words, a process in which one mole of ATP is consumed for every mole of XY produced is energetically less efficient than one in which a smaller amount of ATP is required. This idea can be applied in an exactly analogous way to processes which produce ATP. The basic concept is that the free energy of hydrolysis of ATP is the fundamental "energy" currency of living tissue. This leads to a subsidiary conclusion, often stated in textbooks, that the reason why degradative processes proceed via a large number of intermediate steps, none of which are associated with large standard free energy changes, is that the total free energy change must be liberated in small packets, thus avoiding dissipation as heat.

This "energetic" view of the function of ATP appears to be immediately supported by the fact that ATP breakdown is intimately involved in active transport and in the ultimate step of muscle contraction. In these cases it would be said that the free energy of hydrolysis of ATP is used directly to do osmotic or mechanical work, respectively.

In earlier criticisms of the theory it was pointed out (Hill & Morales, 1951; Gillespie, Maw & Vernon, 1953; Morales, Botts, Blum & Hill, 1955) that the use of a special symbol (~) for a "high-energy bond" is, at best, unfortunate since it is an elementary point that free energy changes are associated with reactions and not with particular reactants. Furthermore, it would appear to be more appropriate to discuss *actual* rather than standard free energy changes since the latter represent, in an important sense, merely an alternative form of equilibrium constants. However, we shall not pursue this since it is our view that the "energetic" interpretation of the function of ATP is totally erroneous and that free energy changes, either actual or standard, are of little relevance.

Since Lipmann's original paper, the preoccupation with standard free energy changes has continued and has led to some curious conclusions. As an example, a paper entitled "Thermodynamics of living systems" (Eyring, Boyce & Spikes, 1960) is worth quoting. In this the authors note that glucose-

6-phosphate is formed *in vivo* by phosphorylation of glucose by ATP. The reason for this is stated to be that the direct phosphorylation of glucose by inorganic phosphate is associated with a positive standard free energy change (+3·0 kcal/mol) and is, therefore, "energetically impossible". In the next paragraph, however, the reaction between phosphorylcreatine and ADP giving ATP and creatine, which is stated to be associated with a standard free energy change of *only c.* 3·5 kcal/mol, is said to be "freely reversible". It is further stated that since −4·8 kcal/mol of free energy is "liberated" in the formation of glucose-6-phosphate (the sum of +3 and −7·8 kcal, the latter being the then accepted standard free energy of hydrolysis of ATP) the reaction is "inefficient energetically speaking". Later in the same chapter, in discussing the same reaction, it is stated that "biochemical arithmetic of this type is a completely invalid application of the free energy concept".

The standard free energies of hydrolysis of a number of phosphate esters are listed in Table 1 (Atkinson & Morton, 1960); the values having as little reliability as that for ATP (see Appendix). However, the important point is that no discontinuity exists. The division of phosphate esters into "high-energy" and "low-energy" compounds depending on whether they appear in the list above or below ATP is, therefore, purely arbitrary. This point was recognized by Klotz (1957) who, in order to emphasize that standard free energies of hydrolysis are not quantities of energy stored in bonds or in compounds, suggested that phosphate esters should be classified according to

TABLE 1

Standard† free energies of hydrolysis of some phosphate esters at pH 7 and 25°, *in the presence of* 0·01 M *magnesium ions*

	$-\Delta G^\circ$ (kcal/mol)
Phosphoenolpyruvate	12·8
Phosphorylcreatine	10·5
Acetyl phosphate	10·1
ATP (α-phosphoanhydride)‡	8·0
ATP (β-phosphoanhydride)§	6·9
Inorganic pyrophosphate	6·6
ADP	6·4
Glucose-1-phosphate	5·0
Fructose-6-phosphate	3·1
Glycerol-1-phosphate	2·3

† By convention, the activities of all components *except the hydrogen ion* are taken as unity. The hydrogen ion activity is taken as 10^{-7}.

‡ $ATP + H_2O = AMP + H_4P_2O_7$.

§ $ATP + H_2O = ADP + H_3PO_4$.

their "group transfer potential", i.e. their ability to donate a phosphate group to a suitable acceptor. This sensible suggestion was unfortunately nullified by an unwise choice of the standard phosphate acceptor, i.e. water, which entailed that the numerical values for the group transfer potential were the same as for the standard free energies of hydrolysis. The error of supposing that the latter represented energy stored in the donors, therefore, continued. The choice of water as the standard acceptor is also bad on the grounds that, as we shall see later, hydrolysis is a forbidden reaction in a metabolic sequence. A better choice of standard phosphate acceptor would have been ADP; esters capable of phosphorylating it would then have positive potentials and, conversely, those esters formed from ATP would have negative potentials. An additional virtue of this scale is that the transfer potential of ATP would be zero.

However, it must be remembered that the arrangement of phosphate esters in order of decreasing standard free energies of hydrolysis or of decreasing group transfer potential (whatever choice is made of standard phosphate acceptor) is merely to state the direction of transphosphorylation in closed systems. This has little to do with what happens *in vivo*.

2. Distinction between Energy linked and Chemically linked Reactions

It has been pointed out that the generalized synthesis of equation (3) may be written as the sum of the direct synthesis of XY [equation (5)] and the hydrolysis of ATP [equation (4)]. However, in a number of cases it has been shown that in the biosynthetic path a phosphorylated (or rarely a pyrophosphorylated) intermediate is formed between XOH and ATP in a step catalysed by a phosphokinase. The phosphate group so transferred is ultimately eliminated under the influence of a phosphorylase. The two reactions are represented by equations (6) and (7).

$$XOH + ATP = XOPO_3H_2 + ADP \tag{6}$$

$$XOPO_3H_2 + YH = XY + H_3PO_4 \tag{7}$$

There is no doubt that the two sets of equations, i.e. (4), (5), (6) and (7), are *thermodynamically* equivalent. However, they are equivalent in no other sense. If XOH, ATP and YH are allowed to interact in a closed system in the presence of enzymes catalysing reactions (6) and (7) then the net chemical transformation will be described approximately by equation (3), that is, for every mole of ATP which disappears one mole of XY will be generated (ignoring the finite concentration of the intermediate $XOPO_3H_2$ necessarily present at equilibrium). If, on the other hand, enzymes catalysing reactions (4) and (5) are added to the same initial system then the two reactions will

proceed independently of each other and the equilibrium concentrations of products will be, ignoring activity effects, exactly the same as if the two reactions were allowed to take place in two unconnected vessels. In other words whereas equations (6) and (7) represent an *actual* route to the synthesis of XY, equations (4) and (5), as they stand, do not. The reason is obvious enough: there is no link between reactions (4) and (5). Reactions (6) and (7) are, however *chemically-linked* in that one of the products of reaction (6) is a substrate for reaction (7). Any set of reactions describing a real biosynthetic process will exhibit this chemical-linkage in that one or more of the products of any given reaction will be substrates for the next. This is why taking reactions (6) and (7) only as a model of a biosynthetic process involves no loss of generality.

There is a further important point: if to a system in which reactions (6) and (7) are occurring, i.e. one in which XY is actually being synthesized at the expense of ATP, enzymes catalysing reactions (4) and (5) are added, the system will tend to move towards the state represented by the two independent equilibria (4) and (5). In other words, reactions (4) and (5) would effectively uncouple the linkage between reactions (6) and (7) and synthesis of XY would be prevented. Not only, therefore, is the hydrolysis of ATP a reaction which cannot contribute to a biosynthetic process mediated by ATP but also *it must not occur* if effective biosynthesis is to take place. It is, presumably, for this reason that ATPase activity *in vivo* is invariably strictly localized and usually particle-bound.

The addition of equations (4) and (5) is simply an arithmetical procedure: it enables the equilibrium constant for reaction (3) to be calculated from those of reactions (4) and (5). The "high-energy" phosphate bond concept thus arises from a confusion between a computational device and a physical process. It is certainly not useful to select one standard free energy of hydrolysis and elevate it to the status of the "driving force" behind metabolic processes, particularly when the chosen reaction is forbidden.

However, since the addition of equations (4) and (5) is a valid thermodynamic procedure there must in principle be a way of carrying out the total reaction such that the equations (4) and (5) are the true components. A possible model for this hypothetical route is that of coupled electrochemical cells in which the cell reactions correspond to equations (4) and (5), i.e. to the hydrolysis of ATP and of XY respectively, the potential of one cell could then be used to reverse the direction of spontaneous change in the other. In such a system the two reactions would be *energy-linked* and it would not be incorrect to say that the free energy of hydrolysis of ATP provided the "driving force" for the other reaction. Furthermore, if such a system were operative, the hydrolysis of one mole of ATP could *in principle* cause the formation of an

integral number of moles, m, of XY, if m cells in which the synthetic reaction occurred were connected in series, given only that $|\Delta G_4^\circ| > m|\Delta G_5^\circ|$. In such a case, the efficiency of utilization of the free energy of hydrolysis of ATP might be a meaningful concept but it should be remembered that there is no known instance of a value of $m > 1$.

In fact, it is unlikely that such systems could actually be constructed since it would be necessary to find two redox reactions whose sum was equivalent to the hydrolysis of ATP. As far as we are aware no such reactions are known. However, the important point is that metabolic processes do not proceed through a set of energy-linked reactions and the peculiarity of the high-energy phosphate bond concept is that it presupposes that they do. In other words the language is appropriate to the wrong model.

At this point, if the above be conceded and if it is agreed that metabolic processes consist of chemically-linked reactions which are such that ATP hydrolysis does not, and indeed must not, occur, then it can still be argued that since a synthetic process such as reaction (3) is *thermodynamically* equivalent to reactions (4) and (5) then it is still necessary that the free energy of hydrolysis of ATP be large and negative otherwise the equilibrium constant for reaction (3) will not be such as to favour the formation of products. As we shall see, when real biological systems are considered this restriction also disappears.

3. Distinction between Open and Closed Systems

So far we have discussed metabolic processes in terms of stoichiometric equations. This is convenient since it allows particular parts of the interlocking network of chemical reactions which constitutes "metabolism" to be discussed independently of the rest. However, it is important to point out that such equations have immediate relevance only to reactions taking place in *closed* systems, i.e. those which exchange energy but not matter across their boundaries. These are the conditions, familiar to the chemist, in which reactions are confined in vessels. But biological organisms are *open* systems, that is, they exchange both energy and matter across their boundaries. It is characteristic of such systems that they tend to steady states in which the amount of matter which enters the system is equal to the amount which leaves. In the simplest case the steady state is achieved when the concentrations of all the intermediates become invariant with time. The importance of distinguishing between closed and open systems is best illustrated by an example. One of the common pathways for the oxidation of glucose is often represented in text-books by the stoichiometric equation (8):

$$C_6H_{12}O_6 + 6\,O_2 + 36\,ADP + 36\,H_3PO_4 = 6\,CO_2 + 42\,H_2O + 36\,ATP \qquad (8)$$

which implies that, for a closed system, for every mole of glucose which disappears 36 moles of ATP are necessarily produced. However, for an organism in steady state the disappearance of one mole of glucose from its *surroundings* would result in no *net* production of ATP. It could be argued that, under such circumstances, the rate of production of ATP is equal to the rate of utilization and that equation (8) still applies to the production of ATP. This may be, but it would be very hard to demonstrate experimentally. If the organism does work on its surroundings it might be thought of interest to calculate the thermodynamic efficiency of the process. To do this it is necessary to identify the net chemical change and to calculate the resultant change in free energy. In so far as the steady state is maintained only glucose, oxygen and the oxidation products enter into the calculation since nothing else changes. More particularly, the breakdown of ATP, even though it may be, e.g. in muscle contraction, the final chemical process before the mechanical changes, does not enter into the thermodynamic account since the chemical potential of ATP remains constant. To interpret the function of ATP in energetic terms is, in these circumstances, therefore, meaningless.

It may be objected that to suppose that an organism exists in a single steady state is an oversimplification since, apart from the possibility of oscillatory behaviour, the organism will tend to different steady states in different environments. This is true but in no sense alters the argument since a thermodynamic account of a system undergoing transition from one steady state to another still requires the identification of those species whose chemical potential changes and information about the extent of those changes. In any case there are reasons for supposing (see below) that the concentration of ATP in most organisms is very effectively buffered and that of all intermediates ATP will show the least change in chemical potential. It, therefore, will contribute least to any thermodynamic account involving free energy changes. Such a situation has been well documented in the case of isolated frog sartorius muscle where significant changes in ATP concentration following contraction can only be demonstrated when all the routes leading to the production of ATP are effectively blocked. (Cain, Davies & Infante, 1962; Cain & Davies, 1962.)

An important characteristic of open systems consisting of a sequence of chemical reactions is that, in general, the individual steps in the sequence will not be at equilibrium in the steady state. This is because the tendency of a particular reaction to move to equilibrium is offset by the feed-in of reactants and the removal of products. Consequently, the behaviour of such systems is not derivative of standard free energy changes (i.e. of equilibrium constants) nor, indeed, of actual free energy changes but depends instead wholly on kinetic parameters, i.e. on the rates of the individual chemical steps. It follows

that for a synthetic reaction of the type given in equation (3) synthesis can occur irrespective of whether the equilibrium constant is highly unfavourable to product formation provided only that products are effectively removed. There is, of course, some limitation: rates depend on the free energies of activation and these cannot be less than the free energy differences between reactants and products. Nevertheless, provided that the standard free energy change associated with a reaction, which is part of a sequence, is not too largely positive the reaction will occur given efficient catalysis. Consequently, whereas the occurrence of reaction (3) in a closed system requires, in an indirect way, that the standard free energy of hydrolysis of ATP be large and negative, its occurrence in a sequence in an open system does not. The standard free energy of hydrolysis of ATP is now irrelevant and whether its value is, in the original Lipmann sense, high, i.e. *c.* -10 kcal/mol, or low, i.e. *c.* -2 kcal/mol, is without significance.

The idea that dynamic chemical systems are controlled by *kinetic* and not by *thermodynamic* factors is by no means new. It has been formulated in many ways but perhaps most clearly by Ingold and his colleagues (Catchpole, Hughes & Ingold, 1948). It arises from the elementary fact that rate processes are dependent on the free energy differences between reactants and the transition states and not upon the free energy differences between reactants and products. Nevertheless, the idea that thermodynamic factors alone control biological processes is implied in many discussions in the area called so misleadingly bioenergetics. For example, Lehninger (1965), in his book *Bioenergetics*, in discussing the tertiary structure of proteins, writes

> "It is one of the most significant conclusions from contemporary research that the extremely elaborate three-dimensional structure of protein molecules, which is so essential for their biological activity and function, is nothing but the logical and inevitable result of the operation of thermodynamic principles. These molecules . . . automatically fold into a biologically specific three-dimensional structure, because this configuration uniquely satisfies thermodynamic principles."

The implication that the native tertiary structures of proteins are the thermodynamically most stable conformations is clearly not generally true since otherwise the routine precautions taken by enzymologists, such as the use of low temperature, obviously aimed at protecting unstable structures would be unintelligible. Furthermore, there are cases (for example, as described by Banks, Doonan, Lawrence & Vernon, 1968) where conformations more stable than the native one can be distinguished electrophoretically. It is, of course, possible that the native conformation is the most stable under the particular local conditions in which the protein is formed but it is more likely that kinetic factors play a controlling part in determining the tertiary structure.

Since open systems are not readily treated by classical thermodynamic concepts attempts have been made to apply the methods of irreversible thermodynamics. For example, a theorem by Prigogine (1945) which states that, under certain conditions, the steady state of an open system is a state of minimum entropy production, has been applied to biological organisms (Prigogine & Wiame, 1946) and leads to the result that an organism tends to a minimum metabolism per unit weight. This theorem, which, even if true, does not appear to have any obvious applications, depends upon the assumption that reaction rate is proportional to thermodynamic driving force, i.e. upon the difference in chemical potential between reactants and products. This is known to be approximately true for reactions close to equilibrium. In other cases reaction rate depends on the free energy of activation, i.e. on the difference between the free energies of the initial and transition states. The point arises as to how close reactions must be to equilibrium for the theorem of Prigogine to be approximately valid. The matter has been examined by Denbigh (1952) who compares the expressions obtained by making rate proportional to the difference in chemical potential or proportional simply to the concentrations of reactants. He concludes that the theorem is approximately true only for systems which diverge from equilibrium by less than RT; i.e. for systems very close to equilibrium. It is now clear that the theorem does not apply to biological systems since the chemical steps involved in these are, in general, far from equilibrium. This is readily appreciated when it is remembered that the equilibrium constant for the oxidation of glucose is of the order of 10^{500}. The number of steps involved in the sequence is less than 50 and it follows, therefore, that most of these must be largely displaced from the equilibrium position.

4. ATP in Some Metabolic Processes

It is now appropriate to illustrate these general principles by discussing some reaction sequences as they occur *in vivo*. The biosynthesis of urea by the Krebs-Hensleit cycle, which conforms to the stoichiometry given in equation (9):

$$\begin{aligned} NH_4^+ + HCO_3^- + H_2O + \text{aspartate}^- + 3\ ATP^{4-} = \text{urea} + \text{fumarate}^{2-} + \\ 2\ ADP^{3-} + \\ AMP^{2-} + \\ 2\ \text{phosphate}^{2-} + \\ \text{pyrophosphate}^{3-} + \\ 4\ H^+ \end{aligned} \qquad (9)$$

is a particularly useful example since it has been the subject of "a complete,

albeit approximate, thermodynamic treatment" (Cohen & Brown, 1960). Furthermore, it represents an "end-process", that is, the final product, urea, is not removed by further chemical transformations but is eliminated from the organism in such a way that its standing concentration is relatively high. Since ammonium ions seem to be relatively toxic to the central nervous system in higher animals it is clearly important that the equilibrium position for reaction (9) should lie far to the right. An "end-process" reaction might be close to its equilibrium position *in vivo* and urea synthesis might, therefore, be an exception to the general principles given above in that the indirect requirement that the standard free energy of hydrolysis of ATP be large and negative, which applies for closed systems, might also apply here. The fact that three moles of ATP are consumed for every mole of urea produced could be taken as support for this view.

As referred to above Cohen and Brown (1960) carried out a thermodynamic analysis of the steps involved, apparently with the intention of predicting which steps involved ATP. It follows from the discussion already given that such a procedure is not possible and this is amply borne out by the conclusions reached. Cohen and Brown discussed reactions (10) and (11)

$$NH_4^+ + HCO_3^- + 2\ ATP = NH_2CO.OPO(OH)_2 + 2\ ADP + H_3PO_4 \qquad (10)$$

$$NH_4^+ + HCO_3^- + ATP = NH_2CO.OPO(OH)_2 + ADP + H_2O \qquad (11)$$

where the former is part of the Krebs-Hensleit cycle as it occurs in liver, catalysed by a synthetase which requires *N*-acylglutamate as cofactor, and the latter occurs in bacteria, catalysed by an enzyme which shows no cofactor requirement except magnesium ions. The two reactions differ in that one requires the breakdown of two moles of ATP whereas the other requires only one. The important point is, however, that both reactions produce the same product, carbamoyl phosphate, in the same molar yield with respect to ammonium and bicarbonate ions. It is clearly not possible to predict which reaction will be part of a particular sequence. In discussing this Cohen & Brown give the value of $\Delta G'^{\circ}_{10}$ as *c.* -2 kcal/mol and estimate $\Delta G'^{\circ}_{11}$ to be *c.* $+5$ kcal/mol. Nevertheless, it is the latter reaction which is known, *in vivo*, to be freely reversible, whereas the former appears to proceed unidirectionally. This illustrates the difference between a reaction taking place in a closed system and, as part of a sequence, in an open system; namely that in the latter case the direction of the flow of matter cannot be predicted from the value of the standard free energy change. Similar difficulty occurs in the discussion of the next step in urea biosynthesis as represented by equation (12).

$$\text{ornithine} + NH_2CO.OPO(OH)_2 = \text{citrulline} + H_3PO_4. \qquad (12)$$

In bacteria the same reaction follows a different stoichiometry [equation (13)].

$$\text{ornithine} + NH_4^+ + HCO_3^- + \text{ATP} = \text{citrulline} + \text{ADP} + H_3PO_4. \quad (13)$$

The standard free energy change associated with reaction (12) is estimated as $\Delta G^\circ_{12} = -1$ kcal/mol. However, Cohen & Brown (1960) were forced to the conclusion that the appropriate liver enzyme catalyses the reaction *only in one direction*—an assertion that requires no comment. Reaction (13), which has a standard free energy change of *c.* +4 kcal/mol, appears to be a non-oxidative pathway to ATP formation in some bacteria but this could not be deduced from energetic considerations nor could it be concluded that such a pathway did not exist in higher animals.

It remains to discuss the equilibrium position of reaction (9). Inspection of the equation shows that, at physiological pH, equilibrium will be far to the right because one of the products, hydrogen ion, is effectively removed. Since the reaction involves the production of no fewer than *four* hydrogen ions, the so-called pH-dependent standard free energy change will be approximately 35 kcal/mol more negative than the true standard free energy change. This factor, which is only indirectly connected with the hydrolysis of ATP, ensures that, *in vivo*, the standing concentration of ammonium ions will be low.

So far the metabolic role of ATP has been discussed in terms of its function as a phosphorylating or pyrophosphorylating agent. However, it may also act as an adenylating agent with the consequent release of inorganic pyrophosphate. The best known example is the first step in protein biosynthesis in which a mixed anhydride of AMP and an amino acid is formed under the control of the appropriate enzyme [reaction (14)].

$$\begin{array}{l} ATP^{4-} + \underset{\displaystyle NH_3^+}{\underset{|}{RCHCOO^-}} = \text{adenosine}-O-\underset{\displaystyle O^-}{\underset{|}{\overset{\displaystyle O}{\overset{\|}{P}}}}-\overset{\displaystyle O}{\overset{\|}{C}}-\underset{\displaystyle NH_3^+}{\underset{|}{\overset{\displaystyle R}{\overset{|}{C}}}}HCOO^- + \text{pyrophosphate}^{3-} \end{array} \quad (14)$$

This process is known as the "activation" of the amino acid and this term is commonly taken to mean that the reaction can be interpreted energetically. This has been done by Lehninger (1965) who asserts that reactions which produce pyrophosphate are subject to a greater "thermodynamic pull" due to subsequent hydrolysis of pyrophosphate by pyrophosphatase. This is necessary, according to Lehninger, since "inorganic pyrophosphate cannot be used directly by the cell to participate in phosphorylation of ADP in glycolysis or oxidative phosphorylation" and, furthermore, since the standard

free energy of hydrolysis of pyrophosphate is said to be *c.* $-6{\cdot}5$ kcal/mol the production of pyrophosphate "ultimately causes the hydrolysis of *two* high-energy phosphate bonds". The implication is, of course, that this amount of free energy is, in some way, "available" for synthetic purposes. The argument is fallacious and it happens to be a bad one even in "high-energy phosphate" terms since, as Lehninger himself points out, the AMP which is necessarily produced also cannot take part in glycolysis or oxidative phosphorylation unless it is rephosphorylated by GTP. What is imagined to be gained by the hydrolysis of pyrophosphate is inevitably lost by the necessity of converting AMP to ADP. The inclusion of some reactions but not others to produce a pseudo-thermodynamic justification for events known to occur *in vivo* is invariably wholly arbitrary.

It appears that the reactions of ATP that produce inorganic pyrophosphates all involve acidic substrates, e.g. amino acids, fatty acids, pantoic acid and, in particular, glutamic acid, which is formed from glutamine and ATP when an amino group is substituted into deamido-NAD or into xanthosine-5′-phosphate to form GMP. AMP is also produced when glutamate condenses with the pteroate skeleton to form folate and when aspartate condenses with citrulline to form argininosuccinate in urea biosynthesis. The mechanistic details of all these reactions are not yet known but it seems possible that they may all involve, like the first step in protein biosynthesis, the production of a mixed anhydride between AMP and a carboxylic acid, in distinction to the phosphate *ester* intermediates involved when ADP is released. If this is the case then a possible reason, of a non-energetic kind, for the elimination of pyrophosphate rather than phosphate can be suggested. The latter would lead to a mixed anhydride between ADP and a carboxylic acid and, as far as we are aware, no such compounds have been described, presumably because they are chemically unstable. As pointed out to us by Dr D. M. Brown they could be expected to undergo intramolecular acyl migration and also to hydrolyse under the influence of intramolecular general-acid catalysis. On the principle that metabolic intermediates must not be unduly reactive chemically (see below) such compounds would not be expected to form part of a metabolic sequence.

5. Muscular Contraction

It has long been supposed that ATP is the "primary energy source" for contraction of skeletal muscle. As such it is often referred to as the "fuel" for muscle function. There is no doubt that this view is largely derivative of Lipmann's "high energy phosphate bond" concept. However, it was noted that there is rather little ATP in muscle; for example frog sartorius contains

about 2 μmol ATP/g compared with 20 μmol phosphorylcreatine/g. More importantly it was found that during normal contraction no change in the ATP concentration could be demonstrated. In writing about this Davies (1964) comments,

> "Numerous experiments . . . have shown that the concentration of ATP remains constant in living muscle during an extended series of contractions and no direct evidence was available that it was involved in muscle contraction. However, the view that it was so involved in this process became widely held when it was realized that adenosine triphosphate was the major energy supply for a whole series of endergonic processes in living matter . . . ."

The implication that although the concentration of ATP does not change it is still the energy source for contraction represents a commonly held but quite erroneous idea since in any energetic (i.e. thermodynamic) accounting only those substances whose chemical potential changes need be considered. It follows that if, during normal contraction, the concentration of ATP does not change then the thermodynamic parameters associated with its formation and breakdown, such as the standard free energy of hydrolysis, are totally irrelevant. The confusion arises because of a failure to distinguish between a thermodynamic and a mechanistic problem. What is required is to show that the interaction of ATP with the actin-myosin system is the ultimate step leading to muscle contraction. Although indirect evidence for supposing that this is true has been known for a long time, namely that ATP, alone of the phosphate esters present in muscle, can, under certain circumstances, cause contraction of glycerinated fibres and also that isolated myosin possesses ATPase activity, direct demonstration of its involvement *in vivo* proved remarkably difficult. The reason is not hard to find. There are four routes to ATP production in muscle (a) glycolysis, (b) oxidative phosphorylation, (c) phosphorylation of ADP by phosphorylcreatine under the influence of phosphorylcreatine kinase (15) and (d) disproportionation of ADP under the influence of myokinase (16).

$$\text{phosphorylcreatine} + \text{ADP} = \text{ATP} + \text{creatine} \tag{15}$$

$$2\ \text{ADP} = \text{ATP} + \text{AMP} \tag{16}$$

The first two can be prevented by treatment with iodoacetate and exclusion of oxygen respectively. Under these circumstances it has long been known that the concentration of phosphorylcreatine falls. Furthermore, as Wilkie and his colleagues (Wilkie & Woledge, 1967) have shown, the relative amounts of the four components of reaction (15) are such that, if the reaction is assumed to

remain sensibly at equilibrium (which it may well do since phosphorylcreatine does not appear to be involved in any other process), conversion of ATP into ADP and inorganic phosphate will cause reaction (15) to move to the right in order to restore the ATP concentration virtually to its initial value. In other words its concentration is effectively *buffered* by reaction (15). The buffering of the ATP concentration is so effective that, in isolated muscle, a decrease in ATP level consequent upon contraction can only be demonstrated when all routes leading to ATP are blocked. This was first achieved by Davies and his colleagues (see Davies, 1964) who found, surprisingly enough, that after treatment of frog sartorius or rectus abdominus muscle with a solution of 1-fluoro-2,4-dinitrobenzene a few contractions could still be elicited and that, under these circumstances, the concentration of phosphorylcreatine remained stationary while that of ATP decreased. Further experiments showed that 1-fluoro-2,4-dinitrobenzene largely prevents the formation of ATP by glycolysis and by oxidative phosphorylation, completely inhibits phosphorylcreatine kinase and partially inhibits myokinase. Only under these extreme conditions can ATP be said to be the energy source for muscle contraction. Under normal conditions its concentration remains in steady state by the action of an elaborate buffer system the significance of which is obscured by "energetic" interpretations of the role of ATP. It follows that, given efficient buffering of the ATP concentration, muscle contraction would work quite satisfactorily even if the standard free energy of hydrolysis of ATP were zero. This is, of course, not open to experimental test but it could be investigated theoretically by analysis of the steady state parameters. The existing evidence can only be interpreted to mean that ATP is essential for the *mechanism* of muscle contraction but irrelevant in considerations of energy changes *in vivo*.

6. Oxidative Phosphorylation

It has long been known that mitochondria are the major site of ATP production and that synthesis is linked to the oxidation of NADH which occurs through a series of reactions usually known as the "electron transport chain". The stoichiometry of the process is thought to conform to equation (17).

$$NADH + H^+ + \tfrac{1}{2}O_2 + 3\,ADP + 3\,H_3PO_4 = NAD^+ + 3\,ATP + 4\,H_2O. \quad (17)$$

However, the actual mechanism by which ATP production is linked to oxidation is not known in spite of intensive research by many groups over the last 30 years. One of the reasons for this lack of progress may be that this field has suffered greater confusion from the inappropriate use of simple thermodynamic parameters than any other.

Equation (17) can be split into the two equations, (18) and (19), representing the oxidation of NADH and the synthesis of ATP respectively.

$$NADH + H^+ + \tfrac{1}{2} O_2 = NAD^+ + H_2O, \tag{18}$$

$$3\ ADP + 3\ H_3PO_4 = 3\ ATP + 3H_2O. \tag{19}$$

In all other metabolic sequences, as we have seen, such reactions would be false components in that they are not chemically linked and would not represent the actual pathway. However, in this case a chemical link between oxidation and phosphorylation has, so far, not been found. This fact has added impetus to discussing the whole process in terms of energy-linked reactions. The sequence by which NADH is oxidized is reasonably well known; it consists of at least seven separate steps involving coupled oxidation-reduction pairs of two types, hydrogen or hydride ion acceptors (e.g. flavoprotein, FP, and possibly, coenzyme Q) and electron acceptors (e.g. the cytochromes, cyt). Only one compound (cytochrome a_3) can interact directly with molecular oxygen. The reactions involved are of four general types represented by equations (20) to (23).

$$NADH + H^+ + FP = NAD^+ + FPH_2, \tag{20}$$

$$FPH_2 + 2\ \text{cyt}\ b^{3+} = FP + 2\ \text{cyt}\ b^{2+} + 2\ H^+, \tag{21}$$

$$2\ \text{cyt}\ b^{2+} + 2\ \text{cyt}\ c^{3+} = 2\ \text{cyt}\ b^{3+} + 2\ \text{cyt}\ c^{2+}, \tag{22}$$

$$2\ \text{cyt}\ a_3^{2+} + \tfrac{1}{2} O_2 + 2\ H^+ = 2\ \text{cyt}\ a_3^{3+} + H_2O. \tag{23}$$

So firmly established is the language in which energy-linked processes are discussed that the standard free energy changes for these reactions are invariably quoted in electrical units: the transformation being made by the well-known relationship

$$\Delta G^\circ = -nFE^\circ$$

where E° is the standard potential of the appropriate electrochemical cell. Now while it is perfectly true that such reactions, unlike most of the other metabolic reactions we have discussed, *could* be made to operate electrochemical cells there is no reason for supposing that they do so in the mitochondria. Electrochemical cells require two electrodes which can act as electron donors or acceptors; the circuit is completed by a solution in which conduction takes place through the movement of ions and by a metallic conductor in which conduction takes place through the movement of electrons. However, the identification of electrodes and of metallic conductors in mitochondria appears not yet to have been made. The next move is to try to determine at which points on the "electron transport chain" the synthesis of ATP occurs by comparing the value of the standard potential for each reaction with the potential required to reverse the hydrolysis of ATP. The model

is again of energy-linked reactions: the synthesis of ATP being accomplished by reversal of the potential of an electrochemical cell whose operation is equivalent to the hydrolysis of ATP. As we have seen there are no two redox reactions whose sum is the hydrolysis of ATP; the model is, therefore, inappropriate. In any case standard potentials, which are simply alternative formulations of standard free energy changes, are irrelevant because the behaviour of steady state systems is not derivative of simple thermodynamic parameters which describe the equilibrium state.

Theories about the actual synthesis of ATP are of two types, those which suppose a chemical link *via* a "high energy" intermediate and those which suppose there is no such link. The supporters of the chemical intermediate (Chance, Lehninger, Slater *et al.*) are at the disadvantage that such an intermediate has not been identified but this has not prevented heated discussion, based on the values of standard potentials, on whether the hypothetical precursor of ATP is itself oxidized (Slater, 1966; Lehninger, Wadkins & Remmert, 1959) or reduced (Chance & Williams, 1959) and whether the formation of ATP "effectively increases the redox potential of the reducing system or decreases the redox potential of the oxidizing system" (Lehninger, 1964). This is a classic example of discussion of a non-existent problem.

Theories which suppose that there is no chemical link between oxidation and phosphorylation have been developed by Green and his colleagues (Harris, Penniston, Asai & Green, 1968) and by Mitchell (1961, 1967, 1968). Green uses coupled electrochemical cells as his model and discusses the process in terms of "transduction" (i.e. change) of electrical energy, derived from electrons flowing down a potential gradient, into chemical energy. The disadvantage with this is that the actual process by which ATP is synthesized is never specified: the proponents of the idea appear to be satisfied when they have convinced themselves that enough "energy" is available though this fact is self-evident since reaction (17) does occur. The nearest to an actual physical model is by analogy; for example, Baum (1968) supposes that the "electrical energy" causes conformational changes in the mitochondrial membrane which are likened to the coiling of a spring. When the spring uncoils ATP or some "high-energy" precursor is automatically synthesized. This cannot be said to constitute a mechanism.

The alternative theory, the so-called chemiosmotic hypothesis of Mitchell, is much more ingenious, even if equally obscure, and deserves credit for the stimulating effect its initial enunciation and subsequent frequent modification has had of research in this field. Mitchell's model is that of a fuel cell rather than of an electrochemical cell. He supposes that the electron transport chain consists of alternating hydrogen and electron carrying components orientated in the membrane in such a way that protons are discharged on one side only,

so generating a proton gradient across the membrane. This generates a "proton motive force" and the inevitable calculation showed that the associated free energy change is greater than the standard free energy of hydrolysis of ATP. This is taken to mean that ATP synthesis *can* now occur. How it occurs is not very clear. In the original version of the theory, the excess of protons on one side of the membrane and the excess of hydroxide ions, formed by reduction of oxygen by electrons, on the other was imagined to drive the reverse of ATP hydrolysis by abstracting hydroxide ions from ADP on one side of the membrane and hydrogen ions from inorganic phosphate on the other. The net result is to abstract the elements of water but precisely how this process would work is by no means clear. In any case the theory came under heavy attack and resulted in a regrettable modification in which the "proton motive force" generates, by some obscure process, an anhydride precursor of ATP. This anhydride is originally formed as a "low-energy" compound but migration across the membrane mysteriously transforms it into a "high-energy" compound the hydrolysis of which reverses the hydrolysis of ATP. The theory is now close to "chemical link" theories and suffers from the disadvantage that the hypothetical anhydride has not been detected.

A recent theory of oxidative phosphorylation put forward by Aldridge & Rose (1969) has certain attractive features. It suggests that a proton flow from the oxidative pathway might be relayed by (paired) stacked histidine residues in the supposedly non-aqueous environment of the mitochondrial membrane, to the site of phosphorylation. The *mechanism* of phosphorylation is admittedly unknown.

In the light of the present discussion which asserts that "energy" considerations are irrelevant in relation to the role of ATP *in vivo* it is possible to examine the problem of oxidative phosphorylation afresh. If it is assumed that there is no chemical link between oxidation and phosphorylation, and, in passing, it should be noticed that this might be biologically advantageous since the stoichiometry is now not necessarily limited to that given by equation (17), it is unnecessary to assume that either electrochemical or fuel cells are involved. Under these circumstances there is only one way in which ATP can be synthesized, namely, by direct esterification of ADP by phosphoric acid and this requires that water be absent. (See Williams, 1969.) This, in fact, is the method first used by chemists in the synthesis of phosphate esters. Chemically it is a poor method since it leads to a variety of products but this difficulty can obviously be overcome by the presence of an appropriate enzyme catalysing only one pathway. What is required is the generation of a non-aqueous microenvironment which contains ADP, inorganic phosphate and an ATPase. Under these conditions ATP synthesis must occur. The exclusion of water from a

microenvironment is no new concept: it appears to occur when chymotrypsin combines with its substrates and contributes to the high nucleophilicity of serine-195 in that enzyme (see Doonan, Vernon & Banks, 1970).

In detail (see Fig. 1) the process might be imagined as follows:

(a) Suppose that a part of the mitochondrial membrane contains ATPase but is normally held in an enzymically inactive, extended configuration by coulombic repulsion between fixed positive charges. Suppose further that a molecule of ADP and of inorganic phosphate become attached to the enzyme and that one of the positive charges is removed by hydride ion transfer from NADH.

(b) The membrane now takes up an infolded configuration thereby extruding water and generating a non-aqueous microenvironment.

(c) A metal cation (M^+) now enters the infolded configuration, perhaps in exchange for a hydrogen ion, and activates the ATPase which then catalyses the formation of ATP.

(d) It is now necessary, in order to avoid violation of the principle of microscopic reversibility, to postulate that the activating cation passes out of the infolded configuration.

(e) The positive charge is now regenerated by hydride transfer to some other part of the electron-transport chain, perhaps to one of the cytochromes.

(f) The membrane takes up the original extended configuration and a molecule of ATP is released.

This theory is consistent with the observations that ion movements and configurational changes in the membrane occur during oxidative phosphoryla-

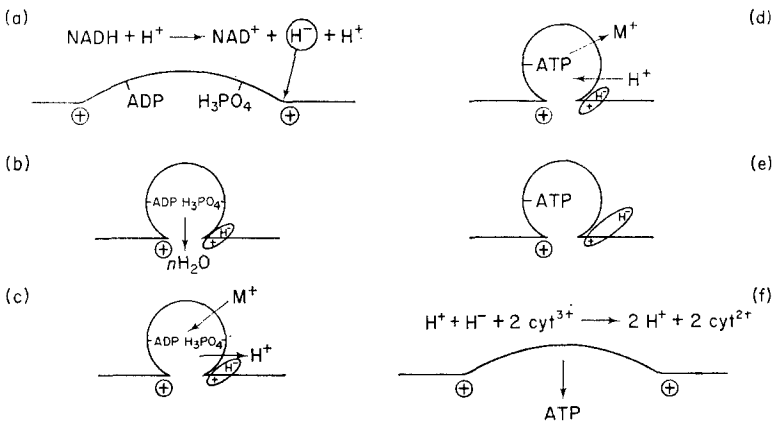

FIG. 1.

tion. It also has the advantage that it postulates no mysterious electromotive or proton motive force and can be discussed without reference to "energetic" factors. The effect of uncouplers could be variously to prevent the change of charge on the membrane or the formation of the infolded configuration or the ion movements necessary to activate and deactivate the enzyme. There is no doubt that the system would work: whether it does, in fact, occur in mitochondria is a matter for experimental test.

7. Conclusion

It remains to inquire the reasons for the unique position of ATP. Obviously it is not possible to decide why ATP and not some similar compound has been selected by evolutionary processes. What can be done, however, is to examine the properties a substance must have in order to play an important role in metabolic sequences. The selection of ATP by evolution is, of course, part of the wider question of why monoesters of phosphoric acid and of condensed polyphosphoric acids are so important in biological processes. Certain properties of these compounds which make them suitable as metabolic intermediates immediately suggest themselves. Firstly, they are all freely soluble in water. Secondly, they are, in general, chemically stable at physiological pH. The advantage of this is obvious: to control chemical pathways involving sequences of enzymic reactions it is necessary that non-enzymic reactions do not proceed at a comparable rate. The relative inertness of phosphate esters led to their neglect by organic chemists for many decades. They were regarded as uninteresting chemical curiosities until the discovery of their importance in metabolic sequences. The "high energy phosphate bond" concept has, however, led to the implied view (commonly stated in textbooks, that, presumably by virtue of their "energy" content, these compounds are unstable. So firmly established is this view that one well-known chemical manufacturer recommends that the disodium salt of phenylphosphate should be stored in a refrigerator. However, from data in the literature (Barnard *et al.*, 1966), it may be calculated that the half-life of phenyl phosphate in solution at pH 7, 20°C, is about 30 years. Thirdly, phosphate esters, particularly those derived from the condensed polyphosphoric acids, undergo ionization with pK_a values in the region of physiological interest and, furthermore, the various ionic species complex readily with divalent metal cations.

The requirement that intermediary metabolites be kinetically stable at neutral pH is the key to the selection of phosphate esters as common intermediates in metabolism. The standard free energy changes for unit steps in closed systems have been shown to be irrelevant in determining the flow of matter through complex sequences and cycles of chemically linked reactions

occurring in open systems but it remains to inquire why many reactions involving the key phosphate ester, ATP, appear to be unidirectional *in vivo*. An answer presents itself by analogy with the situation in muscle. It may very well be that, as is known to be the case in muscle, the level of ATP (and ADP) in all tissues is very effectively buffered, so that the directions of reactions involving ATP could not easily be controlled by fluctuations in substrate concentrations. The properties of enzymes catalysing reactions of ATP suggest that the flow of matter is controlled by means of the enzymic activities since all such enzymes are cation dependent (variously Na^+, K^+, Mg^{2+}, Ca^{2+}) and many are markedly affected by substrate analogues (particularly AMP) and allosteric effectors. The many biosynthetic pathways which depend, *for kinetic reasons*, on the availability of ATP, can then be independently controlled by means of the activity of the relevant enzymes. (It may be noted in passing that in the synthesis of macromolecules, the early steps tend to involve ATP while the later polymerization steps involve other nucleoside triphosphates; CTP in the case of lipid synthesis, GTP for proteins and UTP for polysaccharides, so giving independent control of the three main pathways.)

On the view that the functioning of living systems requires that the key intermediate, ATP, be held in steady state, since it is then available to play its ubiquitous role in synthesis, active transport, muscle contraction, motility etc., it cannot then be regarded as a universal source of energy. Its chemical potential remains constant and it therefore does not enter into any simple thermodynamic considerations. The relative structural complexity of the molecule presumably reflects the necessity for unique recognition by a variety of different enzymes but it is hard to justify the selection of ATP instead of some other nucleoside triphosphate. One thing would appear certain: the standard free energy of hydrolysis of ATP is its least interesting property.

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REFERENCES

ALDRIDGE, W. N. & ROSE, M. S. (1969). *FEBS Letters* **4**, 61.

ATKINSON, M. R. & MORTON, R. K. (1960). In *Comparative Biochemistry*, Vol. 2. (M. Florkin & H. S. Mason, eds.) p. 5. New York: Academic Press.

BANKS, B. E. C. (1969). *Chemy Br.* **5**, 514.

BANKS, B. E. C. (1970). *Atti del seminario di Studi Biologici*, Bari: Adriatica Editrice.

BANKS, B. E. C., DOONAN, S., LAWRENCE, A. J. & VERNON, C. A. (1968). *Eur. J. Biochem.* **5**, 528.

BARNARD, P. W. C., BUNTON, C. A., KELLERMAN, D., MHALA, M. M., SILVER, B. L., VERNON, C. A. & WELCH, V. A. (1966). *J. chem. Soc.* 227.

BAUM, H. (1968). *New Sci.* **38**, 460.

BENZINGER, T. H. (1956). *Proc. natn. Acad. Sci. U.S.A.* **42,** 109.
BENZINGER, T. H. & HEMS, R. (1956). *Proc. natn. Acad. Sci. U.S.A.* **42,** 109.
BENZINGER, T. H., KITZINGER, C., HEMS, R. & BURTON, K. (1959). *Biochem. J.* **71,** 400.
BURTON, K. (1955). *Biochem. J.* **59,** 44.
BURTON, K. & KREBS, H. A. (1953). *Biochem. J.* **54,** 94.
CAIN, D. F. & DAVIES, R. E. (1962). *Biochem. Biophys. Res. Communs* **8,** 861.
CAIN, D. F., DAVIES, R. E. & INFANTE, A. A. (1962). *Nature, Lond.* **196,** 214.
CATCHPOLE, A. G., HUGHES, E. D. & INGOLD, C. K. (1948). *J. chem. Soc.* 13.
CHANCE, B. & WILLIAMS, G. R. (1959). *Adv. Enzymol.* **17,** 65.
COHEN, P. P. & BROWN, G. W. (1960). In *Comparative Biochemistry*, Vol. 2. (M. Florkin & H. S. Mason, eds.) p. 161. New York: Academic Press.
DAVIES, R. E. (1964). In *Muscle.* (W. M. Paul, E. E. Daniel, C. M. Kay & G. Monckton, eds.) p. 49. Oxford: Pergamon Press.
DENBIGH, G. K. (1952). *Trans. Faraday Soc.* **48,** 389.
DOONAN, S., VERNON, C. A. & BANKS, B. E. C. (1970). *Progr. Biophys. biol. Chem.* **20,** 247.
EYRING, H., BOYCE, R. P. & SPIKES, J. D. (1960). In *Comparative Biochemistry*, Vol. 2. (M. Florkin & H. S. Mason, eds.) p. 15. New York: Academic Press.
GEORGE, P. & RUTMAN, R. J. (1960). *Progr. Biophys.* **10,** *2.*
GILLESPIE, R. J., MAW, G. A. & VERNON, C. A. (1955). *Nature, Lond.* **171,** 1147.
GUINODMAN, P. M. (1954). *Biokhimija* **19,** 666.
HARRIS, R. A., PENNISTON, J. T., ASAI, J. & GREEN, D. E. (1968). *Proc. natn. Acad. Sci. U.S.A.* **59,** 830.
HILL, T. L. & MORALES, M. F. (1951). *J. Am. chem. Soc.* **73,** 1656.
KITZINGER, C. & HEMS, R. (1959). *Biochem. J.* **71,** 395.
KLOTZ, I. M. (1957). *Some Principles of Energetics in Biochemical Reactions*, New York: Academic Press.
LEHNINGER, A. L. (1964). *The Mitochondrion*, New York: W. A. Benjamin Inc.
LEHNINGER, A. L. (1965). *Bioenergetics*, New York: W. A. Benjamin Inc.
LEHNINGER, A. L., WADKINS, C. L. & REMMERT, L. F. (1959). *Ciba Fdn Symp.* 130.
LEVINTOW, L. & MEISTER, A. (1954). *J. biol. Chem.* **209,** 265.
LIPMANN, F. (1941). *Adv. Enzymol.* **1,** 99.
MEYERHOF, O. & GREEN, H. (1949). *J. biol. Chem.* **178,** 655.
MITCHELL, P. (1961). *Nature, Lond.* **191,** 144.
MITCHELL, P. (1966). *Nature, Lond.* **212,** 257.
MITCHELL, P. (1968). *Chemiosmotic Coupling and Energy Transduction*, Bodmin, Cornwall, England: Glynn Research Ltd.
MITCHELL, P. & MOYLE, J. (1965). *Nature, Lond.* **208,** 1205.
MITCHELL, P. & MOYLE, J. (1967). *Nature, Lond.* **213,** 137.
MORALES, M. F., BOTTS, J., BLUM, J. J. & HILL, T. L. (1955). *Physiol. Rev.* **35,** 475.
OESPER, P. (1951). In *Phosphorus Metabolism*, Vol. 1. (W. D. McElroy & B. Glass, eds.) p. 523. Baltimore: Johns Hopkins.
PRIGOGINE, I. (1945). *Bull. Acad. r. Belg. Cl. Sci.* **36,** 600.
PRIGOGINE, I. & WIAME, J. M. (1946). *Experientia* **2,** 351.
RACKER, E. (1961). *Adv. Enzymol.* **23,** 323.
ROBBINS, E. A. & BOYER, P. D. (1957). *J. biol. Chem.* **224,** 121.
ROSE, I. A. (1953). *Meth. Enzym.* **1,** 591.
SLATER, E. C. (Ed.) (1966). In *Flavins and Flavoproteins*, **8,** 549. Elsevier, Amsterdam.
SLATER, E. C. (1967). *Eur. J. Biochem.* **1,** 317.
STADTMAN, E. R. (1955). *Meth. Enzym.* **1,** 596.
VERNON, C. A. (1960). In *Size and Shape Changes in Contractile Polymers.* (A. Wasserman, ed). p. 109. Oxford: Pergamon.
VLADIMIROV, G. E., VLASSOVA, V. G., KOLOTILOVA, A. Y., LYZLOVA, S. N. & PANTELEYEVA, N. S. (1957). *Nature, Lond.* **179,** 1350.
WILKIE, D. R. & WOLEDGE, R. C. (1967). *Proc. R. Soc. B* **169,** 17.
WILLIAMS, R. J. P. (1969). *Curr. Topics Bioenerg.* **3,** 79.

APPENDIX

The Standard Free Energy of Hydrolysis of ATP

Although it has been shown that the value of this quantity has no particular significance, it is of interest to examine the validity of the data upon which phosphate esters are classified into "low" or "high" energy compounds. The reaction primarily involved is represented by equation (A1) [equation (4) written with the ionization states appropriate to pH 7·4]

$$ATP^{4-} + H_2O = ADP^{3-} + HPO_4^{2-} + H^+ \qquad (A1)$$

It is worth pointing out that usage of the conventional symbol for standard free energy change, ΔG°, is incorrect in the present context since the standard free energy change refers to conditions in which *all* components are present *at unit activity*. It is made clear (Burton & Krebs, 1953) that on this definition, ΔG°_{A1} (pH independent) is +1 kcal/mol. Noting that *in vivo*, one of the products, H^+, is buffered at *c.* 10^{-7} M and is, therefore, not present at unit activity, the *pH dependent* free energy change is calculated to be −8 kcal/mol ($\Delta G^\circ + RT \ln 10^{-7}$). This convention is universally adopted in discussing standard free energy changes for reactions *in vivo*. In attempting to account for the "high-energy" nature of ATP, discussions centre on the structure of this molecule (e.g. theories of opposing resonance) while the production of a proton in the course of hydrolysis is often ignored. It should be obvious from the above that the production of a proton is the dominant factor in deciding the direction in which equilibrium lies under physiological conditions (George & Rutman, 1960).

The earliest estimates of the standard free energy change were in the range −10 to −14 kcal/mol but were based on imprecise thermochemical measurements (see Oesper, 1951) and are clearly of little value. Equally suspect are estimates involving the use of heats of formations (Burton & Krebs, 1953) since these quantities are an order of magnitude larger than the quantity to be estimated. Most later workers have proceeded by measurement of the equilibrium constants for a series of reactions which sum to give equation (A1). Direct measurement on reaction (A1) has apparently not been attempted presumably on the grounds that the equilibrium lies too far to the right.

There are certain elementary precautions which must be observed in the measurement of equilibrium constants by simple analysis: (a) it is necessary to show that equilibrium has been reached and this is best done by approaching equilibrium from both directions; it is not sufficient simply to add a large quantity of enzyme; (b) the concentrations of all the species present at equilibrium should be independently determined; and (c) conditions of pH, temperature and solvent should be precisely specified. In addition, in order for

the value obtained to be meaningful, it is necessary to work at low concentrations (i.e. $< 0{\cdot}01$ M) in order to minimize activity corrections and to prove that this has been achieved by making measurements over a wide range of substrate concentrations.

No set of data used in the calculation of the equilibrium constant for hydrolysis of ATP so far reported satisfy these criteria. One of the earliest sets (Meyerhof & Green, 1949) used the equilibrium constants for the hydrolysis of glucose and glycerol phosphates measured by catalysis of the reverse reactions by alkaline phosphatase. However, the substrate concentrations used were very high (4 M glucose and 11 M glycerol†), and the data obtained have, therefore, no obvious application.

Robbins & Boyer (1957) measured the equilibrium constant of reaction (24) catalysed by hexokinase,

$$\text{glucose} + \text{ATP} = \text{glucose-6-phosphate} + \text{ADP} \qquad (24)$$

They used what is obviously the analytical technique of choice, namely isotope dilution, thus avoiding the necessity for high substrate concentrations. The effect of variation of Mg^{2+} ions (cofactor for hexokinase) was also investigated. However, no analysis of nucleotides was made and the pH of the solution was not controlled. It was reported that equilibrium was established in twenty minutes in spite of the fact that the values of the equilibrium constant differed significantly depending upon the direction from which equilibrium was approached. The quoted value of the standard free energy change ($\Delta G^0_{24} = -4{\cdot}7$ kcal/mol, 30°C, pH 7, excess Mg^{2+}) is, however, probably not grossly in error. Nevertheless, combination with the value of the standard free energy change for the hydrolysis of glucose-6-phosphate ($-3{\cdot}1$ kcal/mol, syrup, pH 8·5, no Mg^{2+}) obtained by Meyerhof & Green (1949) leads to a value for ΔG°_{A1} ($-7{\cdot}6$ to $-7{\cdot}8$ kcal/mol) which cannot be regarded as reliable.

Reaction (24) has been independently investigated by Russian workers (Vladimirov, Vlassova, Kolotilova, Lyzlova & Panteleyeva, 1957) who also used an isotope dilution technique. Equilibrium was approached from only one direction and the value of the equilibrium constant obtained varied considerably with initial substrate concentration. The final value of the standard free energy change reported ($-3{\cdot}2$ kcal/mol, pH 7·25, excess Mg^{2+}) is considerably lower than the value given by Robbins & Boyer (1957). The hydrolysis of glucose-6-phosphate has also been investigated by an isotope dilution method (Guinodman, 1954) which gave a value for the standard free energy change of $-2{\cdot}45$ kcal/mol. Addition of these two quantities gives $\Delta G^\circ_{A1} = -5{\cdot}6$ kcal/mol: considerably lower than reported

† It is worth pointing out that 11 M-glycerol in water contains 1012 g glycerol/l.

by other workers and much lower than the value originally quoted by Lipmann (1941). This Russian work, which appears to have been largely ignored, must be regarded as providing the best available estimate for the standard free energy of hydrolysis of ATP since the component reactions were both studied by an unequivocal method, i.e. isotope dilution. The corresponding value of the equilibrium constant is 10^{-4} and, if this is correct, the hydrolysis of ATP should be accessible to direct study by isotope dilution techniques if the necessary experimental effort were thought worthwhile.

Equations (25) and (26) also add up to the stoichiometry of equation (A1).

$$\text{Glutamate}^{2-} + \text{ATP}^{4-} + \text{NH}_4^+ = \text{glutamine}^- + \text{ADP}^{3-} + \text{HPO}_4^{2-} + \text{H}^+ \quad (25)$$

$$\text{Glutamine}^- + \text{H}_2\text{O} = \text{glutamate}^{2-} + \text{NH}_4^+ \quad (26)$$

Reaction (25), catalysed by glutamine synthetase from green peas, was studied by Levintow & Meister (1954). These workers used low substrate concentrations, carefully controlled pH, low ionic strength and temperature and analysed for four of these components at equilibrium. Nevertheless, the values of the equilibrium constant obtained differed depending on the direction of approach. The quoted value of the standard free energy change (−4·3 kcal/mol, 37°C) appears to be derived from a somewhat arbitrary choice of the more internally consistent data for the reverse reaction.

Reaction (26), catalysed by glutaminase, has been studied calorimetrically. This method, in which the thermodynamic parameters, ΔG, ΔH and ΔS, are evaluated from the total heat evolved on approach to equilibrium from both directions at different temperatures, is elegant but, as one of the authors points out (Benzinger, 1956), is not valid for substrate concentrations greater than 0·01 M. Nevertheless, Benzinger & Hems (1956), in studying reaction (26), used concentrations of *c.* 0·9 M in ammonia and in glutamate. They then corrected the data by introducing values of mean ion activity coefficients the validity of which cannot be assessed from the available literature. Subsequently (Kitzinger & Hems, 1959; Benzinger, Kitzinger, Hems & Burton, 1959), the value of the standard free energy change obtained (−3·35 kcal/mol, 37°C, pH 7·0) was added to that for reaction (25) (corrected so as to allow for the chelating of the phosphate components with Mg^{2+} ions) to give a value for ΔG°_{A1} of −7 kcal/mol. It does not appear that this value is particularly reliable.

Another set of reactions which have been used are those given in equations (27), (28) and (29).

$$\text{Acetyl-CoA} + \text{H}_2\text{O} = \text{OAc}^- + \text{CoA–SH} \quad (27)$$

$$\text{OAc}^- + \text{ATP} = \text{acetyl phosphate} + \text{ADP} \quad (28)$$

$$\text{acetyl phosphate} + \text{CoA–SH} = \text{acetyl–CoA} + \text{phosphate} \quad (29)$$

However, the relevant papers (Rose, 1953; Stadtman, 1955; Burton, 1955) give too little experimental detail for the reliability of the data to be assessed.

From the foregoing it is apparent that the value of the standard free energy of hydrolysis of ATP is not known with any certainty. The best value (−5·6 kcal/mol) appears to be that reported by the Russian workers. The most favoured value (−7 kcal/mol), on the other hand, depends on rather poor data.