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Review

Role of water in the energy of hydrolysis of phosphate compounds – energy transduction in biological membranes

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I. Introduction

Different forms of energy are interconverted in biological systems. For this purpose, the cell uses phosphate compounds as the common currency of energy exchange [1]. The hydrolysis of these compounds is catalyzed by enzymes that are capable of energy transduction. These enzymes use the chemical energy derived from the cleavage of phosphate compounds such as ATP, acetyl phosphate and pyrophosphate to perform mechanical work, to build up ionic gradients across membranes or to promote the synthesis of new molecules.

Until recently, it was thought that the energy of hydrolysis of the different phosphate compounds would be the same regardless of whether they were in solution in the cytosol or bound to the enzyme surface, and that energy would be released and become available to the enzyme only after the phosphate compound had been hydrolyzed. In this view, energy was not required for binding reactants, nor for release of products from the enzyme surface. The sequence of events in the process of energy transduction was thought to be: (1) the enzyme binds the phosphate compound; (2) the phosphate compound is hydrolyzed and energy is released; (3) the energy derived from the cleavage of the phosphate bond is absorbed by the enzyme; (4) the enzyme uses the

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energy absorbed to perform work; and (5) the products dissociate from the enzyme.

Data obtained in different laboratories during the past two decades have led to a different view. During this period it was discovered that the energy of hydrolysis of the different phosphate compounds varies greatly depending on whether they are in solution or on the enzyme surface (Table I). Studies of the catalytic cycle of different enzymes indicate that energy becomes available for the enzyme to perform work before the cleavage of the phosphate compound. In this new view, the sequence of events for the hydrolysis of a phosphate compound is: (1) the enzyme binds the phosphate compound; (2) the enzyme performs work without the phosphate compound being hydrolyzed (this is accompanied by a decrease in the energy level of the phosphate compound; the presence of the phosphate compound allows the enzyme to assume a new conformation and in the transition, work can be performed); (3) the phosphate compound is hydrolyzed in a process which involves relatively small energy change; and (4) the products of hydrolysis dissociate from the enzyme. In the reverse process, phosphate compounds such as ATP or an acyl phosphate residue are synthesized on the enzyme surface without the need of energy. Energy is then needed for the conversion of the phosphate compound from 'low energy' into 'high energy'. The conversion is coupled with a conformational change of the enzyme. In this crucial step, different forms of energy are interconverted.

The changes in the binding environments of the catalytic site responsible for the change in energy level of phosphate compound are not clearly understood at present. There is experimental evidence which suggests that one important factor is change in water activity at the catalytic site of the enzyme. According to the solvation energy concept proposed by George and co-workers [24], such a change in water activity at the catalytic site could account for a significant change in the free energy of hydrolysis of a molecule.

The two main lines of experimental evidence for a correlation between processes of energy transduction and water activity are:

(1) In aqueous mixtures of different organic solvents, the energy of hydrolysis of pyrophosphate or ATP is similar to that measured on the surface of enzymes and is significantly smaller than that measured in totally aqueous solutions (Tables I and II).

(2) The first step in the direction of synthesis of a high-energy bond is facilitated by the presence of organic solvents. Different membrane-bound enzymes are able to synthesize ATP from ADP and P_i when an ionic gradient is formed across the membrane. For these enzymes we now know of conditions that allow us to measure, in the absence of a transmembrane gradient, the initial steps of the catalytic cycle which culminates in the synthesis of ATP. For both the Ca^{2+}-ATPase and the ATP synthase of mitochondria and chloroplasts it has been shown that the formation of a transmembrane ionic gradient is accompanied by a decrease in the apparent K_m for P_i. This effect can be imitated with the use of organic solvents (Table III). In leaky vesicles or soluble enzymes (no gradient), a decrease in water activity promoted by the addition of organic solvent leads to a decrease in the apparent K_m for P_i to a value that is similar to or even lower than that attained when a gradient is formed across the membrane. For other membrane-bound enzymes, such as the Na^+/K^+-ATPase, it is not yet known whether ionic gradients affect the enzyme affinity for P_i. However, organic solvents have been found to decrease the apparent K_m

TABLE II

Energy of hydrolysis of phosphate compounds at 25 °C in media with different water activities

The data shown in the table are from Refs.: [a] 23, 24, 26, 27; [b] 26, 28–31; [c] 26; [d] 29, 31.

Medium	Phosphate compound	$\Delta G°$ (kcal/mol)
Diluted aqueous solution	ATP	−4.9 to −10.7 [a]
	PP_i	−2.8 to −6.3 [b]
Wet chloroform	ATP	+0.3 [c]
	PP_i	−1.8 [c]
66% (v/v) ethylene glycol in water	PP_i	−0.9 to −3.9 [d]
50% (w/v) poly(ethylene glycol) in water	PP_i	+0.2 to +1.3 [d]

TABLE I

Variability of the K_{obs} for the hydrolysis of phosphate compounds

The values shown in the Table are from Refs.: [a] 2–10; [b] 11–19; [c] 20–22; [d] 23–27; [e] 28–31.

Enzyme	Phosphate compound	At the catalytic site		In solution	
		K_{obs} (M)	$\Delta G°$ (kcal·mol^{-1})	K_{obs} (M)	$\Delta G°$ (kcal·mol^{-1})
Ca^{2+}-ATPase, Na^+/K^+-ATPase	acyl phosphate residue	0.6 [a]	+0.3	10^6 [d]	−8.3
F_1-ATPase, myosin	ATP	0.5 [b]	+0.4	10^4–10^7 [d]	−5.5 to −9.7
Yeast inorganic pyrophosphatase	PP_i	4.5 [c]	−0.9	10^2–4×10^4 [e]	−2.8 to −6.4

TABLE III

Apparent K_m for P_i of enzymes which use the energy derived from ionic gradients for the synthesis of ATP from ADP and P_i

(A) Enzyme phosphorylation by P_i at pH 7.0 [2–5,32–39]. (B) Enzyme phosphorylation by P_i [6,10,40]. (C) In absence of gradient and in presence of organic solvents the data shown refer to the synthesis of 'tightly bound' ATP. In presence of gradient the K_m for P_i refers to the synthesis of ATP measured during oxidative phosphorylation [11–13,16,17,41–44]. (D) $P_i \rightleftharpoons$ ATP exchange (45). (E) $P_i \rightleftharpoons PP_i$ exchange measured in presence of 0.5 mM Mg^{2+}. The K_m values are for the ionic species HPO_4^{2-} [46,47].

Enzyme	Reaction measured	K_m for P_i (M)		Without gradient plus dimethyl sulfoxide	
		without gradient	with gradient	v/v	K_m for P_i (M)
Ca^{2+}-ATPase	A	10^{-2}	10^{-3}	10%	10^{-3}
				20%	$2 \cdot 10^{-5}$
				40%	$7 \cdot 10^{-6}$
Na^+/K^+-ATPase	B	$6 \cdot 10^{-3}$	–	40%	$3 \cdot 10^{-5}$
F_1-ATPase	C	$> 4 \cdot 10^{-1}$	10^{-3}	40%	10^{-3}
Yeast H^+-ATPase	D	$1.4 \cdot 10^{-2}$	–	20%	$4 \cdot 10^{-3}$
Inorganic pyrophosphatase	E	$> 10^{-2}$	$> 10^{-2}$	20%	$3.5 \cdot 10^{-3}$

for P_i in all enzymes tested so far that are involved in processes of energy transduction (Table III).

II. High-energy molecules – theoretical approach

Phosphate compounds with a large free energy of hydrolysis have been described as 'high-energy' molecules. When the hydrolytic reaction of one of these compounds reaches equilibrium, the concentration of products in solution is much higher than that of reactant. The equilibrium constant of the reaction, whose log is proportional to the standard free energy of hydrolysis is determined by dividing the molar concentration of product by the molar concentration of reactant. Thus, the higher the value of the equilibrium constant for the hydrolysis, the higher the energy of hydrolysis of the phosphate compound.

The concept of high-energy compounds has been analyzed primarily from a theoretical viewpoint. Until 1969, interaction of reactant and product with the solvent were not regarded as playing a role in determining the energy of hydrolysis of phosphate compounds. At that time it was thought that intramolecular effects such as opposing resonance, electrostatic repulsions and electron distribution along the P–O–P backbone were the dominant factors contributing to the large negative free energies of hydrolysis of high-energy phosphate compounds such as pyrophosphate and ATP [48–51]. The negative charges on either side of the linkage repel each other, creating tension within the molecule, and the opposing resonance would generate points of weakness along the P–O–P backbone. Thus, it would be easy to cleave the molecule and difficult to bring together the products of the hydrolytic reaction. In these formulations water was ignored or regarded as a continuous dielectric for the purpose of calculating repulsion energies. However, when dissolved in water, the phosphate compounds interact strongly with the solvent (Table IV). The water molecules that organize around the phosphate compound not only shield the charges of the molecule, thus neutralizing the electrostatic repulsion, but also form bridges between different atoms of the molecule, thus reinforcing the weak points generated along the molecule backbone by opposing resonances.

In 1970, George et al. [24] proposed that interaction of reactants and products with the solvent might play a more important role than intramolecular effects. In this view the energy of hydrolysis of a phosphate compound would be determined by the differences in solvation energies of reactants and products. Solvation energy is the amount of energy needed to remove the solvent molecules that organize around a substance in solution. Thus, a more solvated molecule would be more stable, i.e., less reactive, than a less solvated molecule and the equilibrium constant for hydrolysis would have a high value because the products of the reaction are more solvated than the reactant. The solvation energies of orthophosphate and pyrophosphate are shown in Table IV. In totally aqueous medium and depending on the experimental conditions used, the observed standard energy of hydrolysis (ΔG°_{obs}) measured for the hydrolysis of pyrophosphate varies between -2.8 and -6.3 kcal/mol (Table II). This represents a very small fraction of the total solvation energy of either orthophosphate or pyrophosphate (Table IV). The values calculated for solvation energy are not sufficiently accurate to permit a direct estimate of the energy of hydrolysis of a phosphate compound in solution. However, from the values of Table IV it can be inferred that a small change

TABLE IV

Solvation energies

(A) Values calculated by George et al. [24]. (B) Values calculated by Hayes et al. [25].

Molecule	Solvation energy (kcal/mol)	
	A	B
$H_2PO_4^-$	76	55
HPO_4^{2-}	299	219
PO_4^{3-}	637	–
$H_3P_2O_7^-$	87	–
$H_2P_2O_7^{2-}$	134	164
$HP_2O_7^{3-}$	358	–
$P_2O_7^{4-}$	584	–

TABLE V

ΔH of hydrolysis in gas phase and in water

Reaction	ΔH (kcal/mol)	
	gas phase	solution in water
$H_4P_2O_7 + H_2O \rightarrow 2H_3PO_4$	−0.4 [a]	–
$H_3P_2O_7^- + H_2O \rightarrow H_3PO_4 + H_2PO_4^-$	−0.9	−7.3
$H_2P_2O_7^{2-} + H_2O \rightarrow 2H_2PO_4^-$	−75.2	−6.8
$CH_3COOPO_3H_2 + H_2O \rightarrow CH_3COOH + H_3PO_4$	−4.1	−8 to −10
$CH_3COOPO_3H^- + H_2O \rightarrow CH_3COOH + H_2PO_4^{2-}$	+5.4	−8.6
$CH_3COOPO_3^{2-} + H_2O \rightarrow CH_3COOH + HPO_4^{2-}$	+32.5	–
$+C(NH_2)_2NHPO_3H_2 + H_2O \rightarrow C(NH_2)_3^+ + H_3PO_4$	+9.3	
$+C(NH_2)_2NHPO_3H^- + H_2O \rightarrow C(NH_2)_3^+ + H_2PO_4^-$	+96.1	
$+C(NH_2)_2NHPO_3^{2-} + H_2O \rightarrow C(NH_2)_3^+ + HPO_4^{2-}$	+212.4	

[a] Value calculated by Ewig and Van Wazer [52]. All other values shown in the table are from Hayes et al. [25].

in the organization of solvent around the molecules of reactants and products might easily lead to a significant change in the thermodynamic parameters of a reaction.

In 1978, Hayes et al. [25] calculated the energy of hydrolysis of several phosphate compounds in gas phase and compared these values with those measured in water (Table V). In aqueous solution, acetyl phosphate and the N–P bonds in both phosphocreatine and phosphoarginine are of a high-energy nature. However, in the gas phase this is no longer true. On the contrary, the large positive ΔH of hydrolysis indicates that when reactants and products are not solvated, acetyl phosphate and phosphocreatine are more stable than the products of their hydrolysis. From these data Hayes et al. [25] concluded that solvation energies of reactants and products are by far the most important factors in determining the energies of hydrolysis of phosphate compounds that are used for energy storage and transduction in the living cell. The same conclusion has been reached recently by Ewig and Van Wazer [52], who calculated the energy of hydrolysis of pyrophosphate in the gas phase.

III. Pyrophosphate of high and low energy

The simplest known 'high-energy' phosphate compound is pyrophosphate. Analysis of the different ionic species involved in PP_i hydrolysis in media of different water activities suggests that solvation is a critical factor in determining free energy of hydrolysis. In 1965 Stiller et al. [53] carried out direct measurements of the equilibrium of the pyrophosphatase reaction and reported values for the ΔG°_{obs} of −5.8 to −7.4 kcal/mol at 25 °C, pH 7.5 and ionic strength 0.25. In 1969, Alberty [54] calculated the free energy changes for hydrolysis of both ATP and inorganic pyrophosphate at different values of pH and p*Mg* by adding the free energy changes of hydrolysis of several different reactions. In the pH range of 6.0 to 8.0 and in the presence of Mg^{2+} concentrations varying from zero to 5 mM, the values calculated at 25 °C for the ΔG°_{obs} of both pyrophosphate and ATP varied between −5.0 and −9.9 kcal/mol. Later [28–31], direct measurements of the equilibrium of pyrophosphate hydrolysis revealed that K_{obs}, and therefore the ΔG°_{obs}, of pyrophosphate hydrolysis varies greatly depending on the pH and divalent cation concentration in the medium (Fig. 1). However, the ΔG°_{obs} values measured were smaller than those calculated by Alberty [54]. Flodgaard and Fleron [28] reported that at pH 7.4 the ΔG°_{obs} values measured in the absence and in the presence of 40 mM Mg^{2+} were −5.6 to −2.6 kcal/mol, respectively. Similar values were obtained by De Meis [29,31] and by Daley et al. [30] at pH and Mg^{2+} concentrations similar to those used by Flodgaard and Fleron (Fig. 1).

The values of ΔG°_{obs} are related to the total concentrations of all ionic species of pyrophosphate and P_i, including those that are free and those that are in the form of a complex with divalent cations. Table VI shows the ΔG°_{ionic} values calculated from data obtained at different pH values and in the presence of different Mg^{2+} and Ca^{2+} concentrations. Notice that the energy of pyrophosphate hydrolysis varies greatly depending on whether it is the free ionic species of pyrophosphate

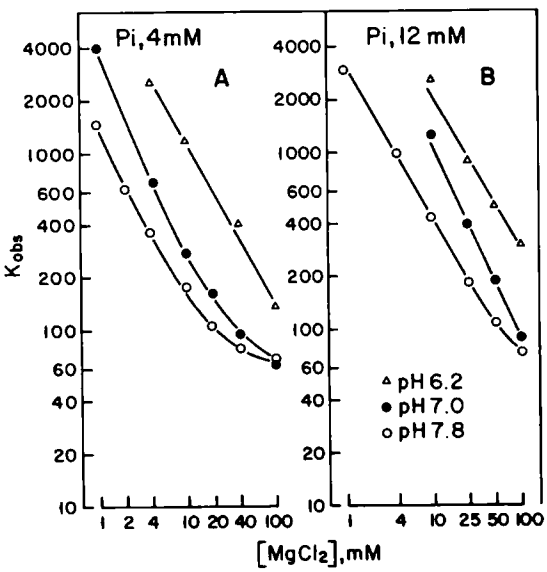

Fig. 1. Effect of $MgCl_2$ on the observed equilibrium constant (K_{obs}) of pyrophosphate hydrolysis measured at different pH values. The assay was performed at 35 °C. The buffers used were 50 mM imidazole (pH 6.2 and 7.0) or 50 mM Tris-HCl (pH 7.8). The P_i concentrations were 4 mM (A) or 12 mM (B). For details see Ref. 29 (reproduced with permission).

TABLE VI

Energy of hydrolysis of different ionic forms of pyrophosphate in totally aqueous medium and in the presence of ethylene glycol

For experimental details, see Refs. 29 and 31.

Reaction	ΔG°_{ionic} (kcal/mol)	
	water	ethylene glycol 66% (w/v)
$HP_2O_7^{3-} + H_2O \rightarrow H_2PO_4^- + HPO_4^{2-}$	−5.4	−2.8
$H_2P_2O_7^{2-} + H_2O \rightarrow 2\ H_2PO_4^-$	−6.3	−3.9
$MgP_2O_7^{2-} + H_2O \rightarrow MgHPO_4 + HPO_4^{2-}$	−2.7	−0.5
$MgHP_2O_7^- + H_2O \rightarrow MgHPO_4 + H_2PO_4^-$	−3.6	−1.5
$Mg_2P_2O_7 + H_2O \rightarrow 2\ MgHPO_4$	−2.8	−1.0
$CaP_2O_7^{2-} + H_2O \rightarrow CaHPO_4 + HPO_4^{2-}$	−1.8	-
$CaHP_2O_7^- + H_2O \rightarrow CaHPO_4 + H_2PO_4^-$	−2.1	-
$Ca_2P_2O_7 + H_2O \rightarrow 2\ CaHPO_4$	−1.5	-

and P_i or their complexes with either magnesium or calcium that are involved in the equilibria studied. A comparison of Tables I and VI suggests that the large difference between the energy of pyrophosphate hydrolysis in solution and that on the enzyme surface could be in part related to the presence of a high magnesium concentration at the catalytic site of inorganic pyrophosphatase. Under physiological conditions, magnesium enters the catalytic site both as magnesium pyrophosphate and as $Mg \cdot P_i$ complexes [21,22]. However, even for the lowest value of ΔG°_{ionic} measured with magnesium in solution (−2.7 kcal/mol), the value in solution is still significantly more negative than that measured for the hydrolysis of pyrophosphate bound to the catalytic site of yeast inorganic pyrophosphatase (−0.9 kcal/mol). Notice in Table VI that the ΔG°_{obs} measured in presence of calcium is less negative than that measured in presence of magnesium. However, calcium does not play a role in the catalytic cycle of the enzyme which is inhibited by calcium ions, and the pyrophosphate bound to the enzyme was detected only in the presence of magnesium and absence of calcium ions [22].

Determinations of the K_{eq} for the hydrolysis of phosphate compounds are usually made in media of ionic composition similar to that of the cell cytosol. In these determinations the concentration of water is not taken into account because the reactants and products are assumed to be present in such low concentrations that the activity of water would be essentially constant in spite of changes in the relative amounts of reactant and products. One of the major differences between bulk solution and the surface of proteins is the activity of the solvent. The water molecules that organize around a protein solution have properties that are different from those of medium 'bulk water' – for example, a lower vapor pressure, a lower mobility and a greatly reduced freezing point [55–58]. Similar changes in the properties of water are observed in mixtures of solvents and water [59–63]. Recently, the K_{obs} for the hydrolysis of pyrophosphate and ATP were measured in mixtures of water and organic solvents (Table II). In these experiments, organic solvents were used as a means of changing the water activity of the medium. The aim was to explore the possibility that water structure could play a role in determining the different values of the K_{eq} for hydrolysis of a phosphate compound observed during the catalytic cycle of enzymes (Table I). The K_{obs} for pyrophosphate hydrolysis decreased when different organic solvents were included in the assay medium, reaching values similar to those attained on the surface of the yeast inorganic pyrophosphatase, between 3.5 and 4.5 M (Figs. 2–4). A decrease in the dielectric constant of the medium does not seem to be the determining factor for the decrease of K_{obs} that is seen in Fig. 2. There is only a slight decrease in the dielectric constant of water after the addition of 30% dimethyl sulfoxide, from 80.4 to 78.6 at 20 °C. A larger decrease in the dielectric constant is observed in the presence of either 60% glycerol or 60% ethylene glycol – from 80.4 to 61.5 and 61.1, respectively [29,31]. However, in the presence of 10 mM $MgCl_2$, dimethyl sulfoxide was nearly as effective as glycerol or ethylene glycol in decreasing K_{obs} for pyrophosphate hydrolysis. A common feature of the different organic solvents used is that they alter the water structure [59–63]. In fact, it was found that there is a linear relationship between the decrease in K_{obs} and the decrease in water activity

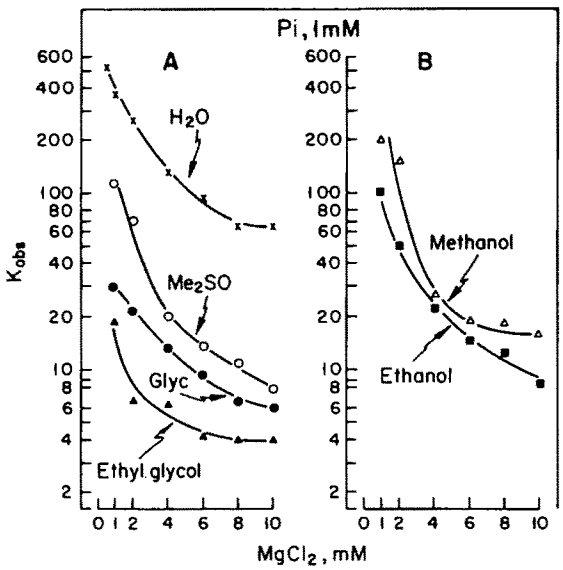

Fig. 2. Effect of organic solvents on the K_{obs} of pyrophosphate hydrolysis. The assay medium consisted of 50 mM Tris-HCl buffer (pH 7.8), 1 mM P_i and the concentrations of $MgCl_2$ shown on the abscissa, without organic solvents (×), 30% dimethyl sulfoxide (○), 60% glycerol (●), 60% ethylene glycol (▲), 30% methanol (△) or 30% ethanol (■). The assay was performed at 35 °C. For details see Ref. 29 (reproduced with permission).

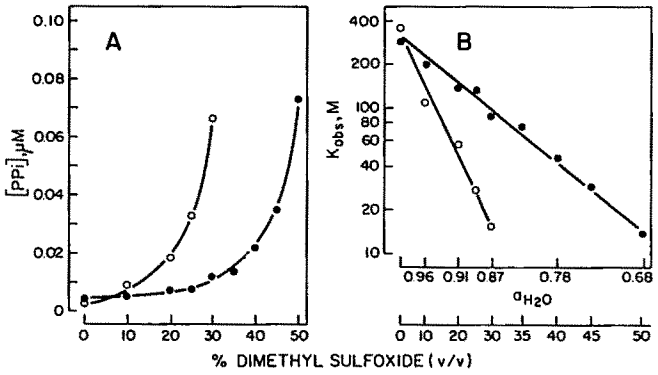

Fig. 3. Effect of dimethyl sulfoxide on the K_{obs} of pyrophosphate hydrolysis. The assay medium consisted of 1 mM P_i, 8 mM $MgCl_2$ and 50 mM of either Tris-HCl or Mops buffer. The final pH in the mixtures was either 7.8 (○) or 7.0 (●). The temperature was 30 °C. In (B) the K_{obs} values were calculated using the equilibrium concentrations of pyrophosphate measured in (A). The water activities (a_{H_2O}) corresponding to the different dimethylsulfoxide concentrations used were determined from vapor pressure values (Ref. 60). For experimental details, see Refs. 29 and 31.

promoted by increasing concentrations of dimethyl sulfoxide (Fig. 3). Another indication that water structure is involved in determining the energy of hydrolysis of pyrophosphate was obtained with the use of poly(ethylene glycol). Ueberreiter [61,62] observed that polymers of ethylene glycol are more effective than ethylene glycol in breaking the water structure, and that this effect increases with increasing molecular weight of the polymer up to about 10000. Accordingly (Fig. 4), it was found that polymers of ethylene glycol were more effective than either ethylene glycol or dimethyl sulfoxide in decreasing the K_{obs} for pyrophosphate hydrolysis [31]. This effect is more pronounced the higher the molecular weight of the polymer (compare Figs. 2, 3 and 4).

The values of K_{obs} measured with the use of organic solvents include contributions from the K_{eq} values for different ionic species of P_i and pyrophosphate (free and in complex form with magnesium). Table VI shows the $\Delta G°$ of different ionic reactions calculated from experimental values obtained in totally aqueous medium and in a solution of ethylene glycol (66% (w/v)). Notice that the organic solvent promoted a decrease in $\Delta G°$ for all the reactions calculated. Wolfenden and Williams [26] measured the distribution of tetraethyl pyrophosphate between water and chloroform. From the distribution coefficients, they estimated the free energies of hydrolysis of pyrophosphoric acid and ATP in wet chloroform (Table II). The energies of hydrolysis thus calculated were significantly smaller than those previously reported for these compounds in totally aqueous medium.

The decreases in the energies of hydrolysis shown in Tables II and VI and in Figs. 2–4 can be interpreted according to the concept of solvation energy. It may be that the alteration of the water structure promoted by the different cosolvents used leads to a slight change in solvation of reactants and products. The high values of solvation energy shown in Table IV indicate that a small change of P_i and/or pyrophosphate solvation would be sufficient to account for the observed decrease in the energy of hydrolysis of pyrophosphate hydrolysis. In this view, the decrease in the energy of hydrolysis can occur in the presence of a large excess of water, as was observed in Figs. 2–4. The reactivity of a given chemical species, such as P_i, would vary depending on its ability to interact with the solvent, and in turn, this interaction would depend on the structure of the solvent. Thus a change of water activity would lead to a change in the activities of P_i and pyrophosphate. Finally, the data of Figs. 2–4 suggest that the difference in energies of hydrolysis between phosphate compounds in solution

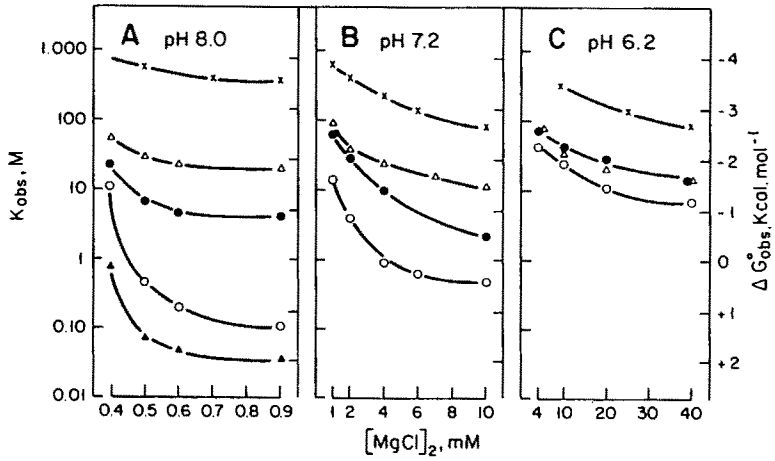

Fig. 4. Effects of pH, $[Mg^{2+}]$ and polymers of ethylene glycol on the energy of hydrolysis of pyrophosphate. The assay was performed at 30 °C. In (A) (▲) the P_i concentration was 0.1 mM. In all other conditions, it was 1 mM. The buffers used were (A) 50 mM Tris-HCl (pH 8.0), (B) 50 mM Mops (pH 7.2), and (C) 50 mM Mes (pH 6.2). For details see Ref. 31 (reproduced with permission). ×, Totally aqueous medium; △, 66% (w/v) ethylene glycol; ●, 50% (w/v) poly(ethylene glycol) 3500 and ○, ▲, 50% (w/v) poly(ethylene glycol) 8000.

and those on the enzyme surface (Table I) could be ascribed to the difference in properties of bulk water and water on the enzyme surface.

IV. The Ca^{2+}-ATPase – acyl phosphate of high and low energy

Vesicles derived from the sarcoplasmic reticulum of skeletal muscle retain a membrane-bound ATPase that can catalyze both the hydrolysis and the synthesis of ATP. Suspended in a medium containing ATP and Mg^{2+}, these vesicles can reduce the Ca^{2+} concentration of the medium from 0.1 mM to less than 0.1 μM. In this process the ATPase uses the chemical energy derived from the hydrolysis of ATP to pump Ca^{2+} into the vesicles and to form a steep Ca^{2+} concentration gradient across the vesicle membrane [4,64–72]. The entire process of Ca^{2+} transport can be reversed. Under appropriate conditions the enzyme can use the energy derived from a Ca^{2+} gradient previously formed across the vesicle membrane to synthesize ATP from ADP and P_i. The synthesis of ATP is coupled to a fast efflux of Ca^{2+} [4,66–76]. The reaction sequence shown in Fig. 5 describes the steps involved in the process of ATP hydrolysis and Ca^{2+} transport [77]. The data supporting this cycle have been discussed in detail in previous reviews [4,68–72]. In the cycle, E and *E represent two distinct conformations of the enzyme. For the hydrolysis of ATP the catalytic cycle of the enzyme is initiated by the binding of Ca^{2+} to a high-affinity site (K_s about 1 μM at pH 7.0) located on the part of the ATPase molecule that faces the outer surface of the vesicles. At pH 7.0, two Ca^{2+} ions bind to the enzyme molecule in a cooperative process [78–80]. After the binding of Ca^{2+}, a carboxyl group of an aspartic acid residue located in the catalytic site of the enzyme is phosphorylated by ATP, forming an acyl phosphate residue [81]. In totally aqueous medium, the energy of hydrolysis of an acyl phosphate residue is practically the same as that of the γ-phosphate of ATP. Accordingly, the equilibrium constant of reaction 3 in Fig. 5 is about 1, and this step of the cycle is fully reversible [65] even in the absence of a transmembrane ionic gradient. After phosphorylation, the enzyme undergoes a conformational change and the Ca^{2+} binding site which was facing the outer surface of the vesicle now faces the vesicle lumen. This is associated with an increase in the K_s from 1 μM to 1 mM, which permits the release of the bound Ca^{2+} in the vesicle lumen (reaction 5 and Refs. 82–86).

In the translocation step (reaction 4 in Fig. 5), different forms of energy are interconverted. During the conformational changes of the enzyme there occurs simultaneously a decrease in the enzyme affinity for Ca^{2+} (binding energy) and a decrease in the energy of hydrolysis of the phosphoenzyme (chemical energy). Thus, Ca^{2+} is translocated across the vesicle membrane *before* the hydrolysis of the acyl phosphate residue. Between the two phosphoenzyme forms $2Ca \cdot E \sim P$ and *E-P, only the acyl phosphate of the enzyme form $2Ca \cdot E \sim P$ has a large free energy of hydrolysis. There seems to be no energy release when the acyl phosphate of *E-P is hydrolyzed (reactions 6 and 7). This was deduced from the finding that in the reverse process, the enzyme form *E can be phosphorylated by P_i without the need of energy. This was discovered by studying the reversal of the Ca^{2+} pump. When the synthesis of ATP by the Ca^{2+}-ATPase was first described, it was thought that the energy derived from the Ca^{2+} gradient would be used to phosphorylate the enzyme by P_i, i.e., for the reversal of reactions 6 and 7 in Fig. 5 [76,87–89]. This interpretation was based on the assumption that an acyl phosphate residue would have a large energy of hydrolysis regardless of whether it was in solution in the cytosol or bound to the enzyme surface. Shortly afterwards, it was found that the ATPase of leaky vesicles can be phosphorylated by P_i to form an acyl phosphate residue in the absence of a transmembrane Ca^{2+} gradient [2–5,32–39,90–93]. The phosphoenzyme formed in the absence of a Ca^{2+} gradient has been referred to as having 'low energy' because it is formed spontaneously and does not transfer its phosphate to ADP [4,32]. However, following the addition of a high concentration (more than 1 mM) of Ca^{2+} to the medium, the phosphoenzyme of leaky vesicles (no gradient) is converted from a form which cannot transfer its phosphate to ADP into a a form which can, i.e., from a 'low-energy'

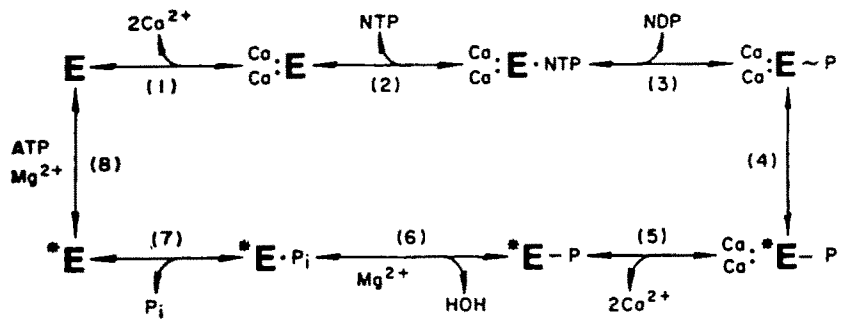

Fig. 5. Reaction sequence of the Ca^{2+}-ATPase of sarcoplasmic reticulum [4,71].

into a 'high-energy' form. This conversion is promoted by the binding of Ca^{2+} to the enzyme and is represented by reversal of reactions 5 and 4 in Fig. 5 [32–34, 77,82,83,90,94]. On the basis of these data, we now know of experimental conditions which allow us to move the different intermediary steps of the catalytic cycle of the enzyme in the direction of ATP synthesis. Thus, either a continuous $P_i \rightleftharpoons$ ATP exchange or a single cycle of net synthesis of ATP can be obtained in the absence of a transmembrane Ca^{2+} gradient [33,34, 77,82,83,90,94–96]. The net synthesis of ATP can be promoted by a variety of perturbations, including Ca^{2+} [33,34,37,90,94], pH [34,94] and water activity jumps [33,34,37].

The first inference that water might be involved in the process of energy transduction catalyzed by the Ca^{2+}-ATPase was based on the calculations of the energy of hydrolysis in gas phase reported by Hayes et al. (Table V and Ref. 25). A crucial point for the understanding of the cycle is how the energy of hydrolysis of the same chemical species, an acyl-phosphate residue, varies when the enzyme changes its conformation (reaction 4 in Fig. 5). The values in Table V show that the acyl-phosphate residue of acetyl phosphate and the phosphoanhydride bond of pyrophosphate have similar energies of hydrolysis in water, but that there is a large difference between the corresponding energies of hydrolysis when the reactants are not solvated. Extrapolating from these calculations to the case of the Ca^{2+}-ATPase, the possibility was raised that in the *E conformation the catalytic site of the enzyme is hydrophobic so that P_i and the aspartic acid would interact as if in a gas phase [33]; the ΔH for the hydrolysis of an acyl phosphate residue would have a positive value, and the reaction would occur spontaneously. In this case the major thermodynamic barrier for the formation of the acyl phosphate residue would be not the formation of the covalent bond (reaction 6 in Fig. 5) but the binding of P_i to the enzyme (reaction 7), i.e., the partitioning of a hydrophilic ion from the assay medium into the hydrophobic environment of the catalytic site. Factors facilitating this partition should also facilitate the phosphorylation of the enzyme by P_i. The phosphoenzyme *E-P formed from P_i would not be able to transfer its phosphate to ADP because of the large difference in the ΔH of hydrolysis of the acyl phosphate and the pyrophosphate residue of ATP in gas phase (Table V). The binding of Ca^{2+} to the enzyme promotes a conformational change in the protein [68,71,72] permitting the entry of water into the catalytic site with subsequent solvation of both the acyl phosphate residue and ADP. As a result, the ΔH values for the hydrolysis of the acyl phosphate and of the pyrophosphate residue would become equal and the synthesis of ATP would proceed spontaneously. Experimental conditions which reduce the entry of water into the catalytic site should also impede the synthesis of ATP. According to this hypothesis the existence of high-energy and low-energy forms of the phosphoenzyme would be related solely to the distribution of water into the catalytic site.

This hypothesis was tested [33–35,37] by measuring the phosphorylation of the enzyme by P_i and the synthesis of ATP in the presence of various organic solvents (dimethyl sulfoxide, glycerol and *N*,*N*-dimethylformamide). In aqueous mixtures, these solvents markedly increase the partition coefficient of P_i from the aqueous medium into an organic phase containing isobutanol and benzene [33]. The phosphorylation of the enzyme by P_i demonstrates a saturation behaviour, indicating the occurrence of a phosphate-enzyme complex prior to the phosphorylation reaction [4];

$$*E + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} *E \cdot P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} *E - P + HOH$$

If the catalytic site of the enzyme is hydrophobic, then the partitioning of P_i from the assay medium into the catalytic site (k_1/k_{-1}) should be facilitated when the difference in hydrophobicity of these two compartments is decreased by the addition of organic solvent. The solvent should promote a large decrease in the apparent K_m for P_i. Accordingly, Table III shows that replacing 40% of the water in the assay medium by dimethyl sulfoxide results in a large decrease in the apparent K_m for P_i. The phosphoenzyme formed in the presence of 40% dimethyl sulfoxide is not converted to a high-energy form after the binding of Ca^{2+} to the enzyme, i.e., there is no synthesis of ATP after the addition of Ca^{2+} and ADP to the medium (Fig. 6). The inhibition of ATP synthesis seems to be related to the decrease in water activity that is caused by the presence of the organic

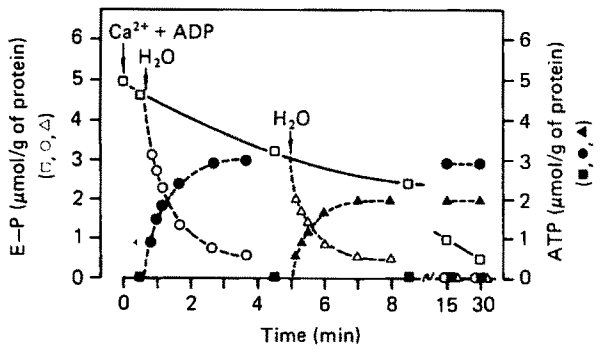

Fig. 6. Inhibition of ATP synthesis by dimethyl sulfoxide. The enzyme was phosphorylated in a medium containing 30 mM Tris-maleate buffer (pH 8.0), 0.05 mM EGTA, 5 mM $MgCl_2$, 1 mM [^{32}P]P_i and 40% (v/v) dimethyl sulfoxide; 15 min after the addition of enzyme, ADP and Ca^{2+} were simultaneously added to final concentrations of 0.15 and 0.40 mM, respectively. In a parallel set of experiments, after the addition of ADP and Ca^{2+}, 0.1 ml of the mixture was diluted in 1.9 ml of a solution of identical composition except that P_i and dimethyl sulfoxide were omitted. In the figure this is represented as (↓ H_2O). Open symbols are radioactive phosphoenzyme and closed symbols are ATP synthesis: □, ■, 40% (v/v) dimethyl sulfoxide; ○, ●, △, ▲, after dilution of dimethyl sulfoxide from 40% to 2%. For details, see Ref. 33 (reproduced with permission).

solvent in the medium. A rapid cleavage of the phosphoenzyme coupled with a stoichiometric synthesis of ATP was observed if, after the addition of ADP and Ca^{2+}, the dimethyl sulfoxide concentration was suddenly decreased from 40% to 2% [33–35,37].

The hypothesis proposed states that high- and low-energy forms of the phosphoenzyme are correlated with the availability of water at the catalytic site of the enzyme. The experimental results are consistent with this hypothesis. As predicted, phosphorylation of the enzyme by P_i is facilitated when the hydrophobicity of the medium is increased by the addition of organic solvents and, after the addition of Ca^{2+}, the phosphoenzyme is able to transfer the phosphate to ADP only if the water activity of the medium is increased by dilution of the organic solvent. These data have been reproduced in different laboratories [35–39] and the same approach has been applied to other systems. As shown in Table III, there is a significant decrease in the apparent K_m for P_i of several enzymes involved in energy transduction when organic solvents are included in the assay medium. Finally, it was recently found [97] that a decrease in the apparent K_m for P_i, similar to that measured with organic solvents, can also be obtained with the use of the methylamines glycine betaine and trimethylamine. These methylamines are found in different tissues. The effect of either organic solvent or methylamines is cancelled by adding an appropriate concentration of urea [97,98].

Other data which support the proposal that the change in the energy of hydrolysis of the phosphoenzyme is related to a hydrophobic-hydrophilic transition during the catalytic cycle are as follows.

(1) Measurements at different temperatures of the rate constants for enzyme phosphorylation by P_i and for phosphoenzyme hydrolysis (reactions 6 and 7 forwards and backwards in Fig. 5) reveal that the addition of organic solvent to the medium promotes a significant change in both the entropy and the enthalpy of reaction 6. The addition of 40% dimethyl sulfoxide to the medium leads to an increase in the standard enthalpy for hydrolysis of the 'low-energy' phosphoenzyme from -1.9 to $+18.0$ kcal. Note that the value of the standard enthalpy measured in the presence of dimethyl sulfoxide is similar to that calculated by Hayes et al. [25] for the hydrolysis of acetyl phosphate in gas phase (Table V).

(2) Experiments performed with fluorescent probes indicate that there is a change in solvent accessibility at the catalytic site of the Ca^{2+}-ATPase during the catalytic cycle [99–101].

(3) Andersen et al. [102] observed an increase in the hydrophobic labeling of the sarcoplasmic reticulum ATPase with the photoactivatable reagent trifluoromethyl [^{125}I]iodophenyldiazarine when the enzyme was stabilized in the *E form (which binds P_i) as compared with the E form (which binds ATP). The preferential hydrophobic labeling was found to be located on the same tryptic fragment as the catalytic site of the enzyme.

(4) As discussed above (Table II), there is a significant decrease in the observed energy of hydrolysis of both pyrophosphate and ATP when the water activity of the medium is decreased by the addition of organic solvents.

V. The Na^+/K^+-ATPase

What follows is a brief discussion of the catalytic cycle of the Na^+/K^+-ATPase, another example in which the energy of hydrolysis of a phosphate compound varies during the catalytic cycle. Excellent reviews recently published discuss in detail the many experimental data available for this system [8,9,103,104]. Like the Ca^{2+}-ATPase of sarcoplasmic reticulum, the Na^+/K^+-ATPase can catalyze either the hydrolysis of ATP or, in the reverse direction, both the synthesis of ATP from ADP and P_i and a continuous $P_i \rightleftharpoons$ ATP exchange. The reversal of the Na^+/K^+ pump can be measured in the absence of a transmembrane ionic gradient [6–8,10,40,105]. During the catalytic cycle of the Na^+/K^+-ATPase, two different forms of phosphoenzyme can be detected; these are represented as $Na \cdot E_1 \sim P$ and $Na \cdot E_2$-P in Fig. 7. In both phosphoenzymes, a carboxyl group of an aspartic acid residue located in the catalytic site is phosphorylated, forming an acyl phosphate residue. The enzyme can be phosphorylated either by ATP in reaction 1 forwards (Fig. 7) or by P_i during reversal of reaction 3. The phosphoenzyme E_2-P is referred to as a 'low-energy' form because it is not able to transfer its phosphate to ADP and it is spontaneously formed from P_i in the absence of a transmembrane gradient, i.e., without any apparent source of energy. The phosphoenzyme $Na \cdot E_1 \sim P$ is referred to as a 'high-energy' form because it can transfer its phosphate to ADP, leading to the synthesis of ATP. The newly synthesized ATP does not remain tightly

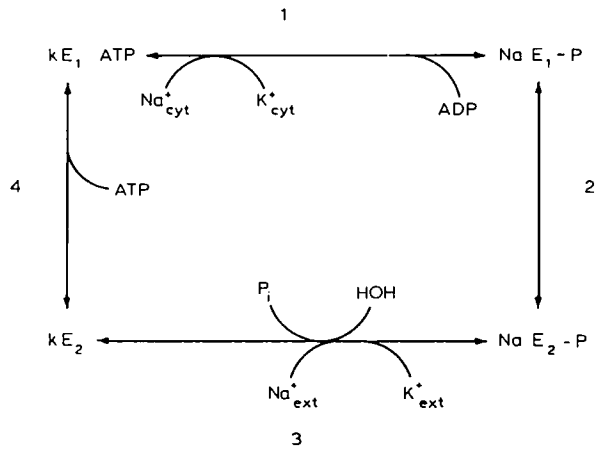

Fig. 7. Reaction sequence of the Na^+/K^+-ATPase [8].

bound on the enzyme surface, but readily dissociates into the assay medium. Conversion of the phosphoenzyme from 'low-energy' into 'high-energy' forms can be achieved in the absence of a transmembrane gradient [6,7,10,105] by adding a high concentration of Na^+ to the medium (about 600 mM NaCl at pH 7.0). This conversion is associated with a conformational change of the enzyme, translocation of Na^+ through the membrane and modification of the enzyme affinity for Na^+. Therefore, as for the Ca^{2+}-ATPase, conformational, binding and chemical energies are interconverted during the translocation step (reaction 2 in Fig. 7) *and before* the hydrolysis of the acyl phosphate residue.

In the absence of a transmembrane gradient, a 200-fold decrease in the P_i concentration needed for half-maximal enzyme phosphorylation is observed when 40% dimethyl sulfoxide is added to the assay medium (Table III). The phosphoenzyme formed in the presence of a low concentration of organic solvent can transfer its phosphate to ADP to form ATP when 400 mM NaCl is added to the medium. The synthesis of ATP is inhibited when the concentration of dimethyl sulfoxide is raised to 60% [10]. As discussed for the Ca^{2+}-ATPase, the data obtained with organic solvent might be interpreted according to the proposal that the conversion of the phosphoenzyme from a 'low-energy' into a 'high-energy' form is related to a hydrophobic–hydrophilic transition. The concentration of dimethyl sulfoxide needed to block the phosphate transfer from the phosphoenzyme to ADP varies depending on the enzyme used. For the Ca^{2+}-ATPase the transfer is completely blocked in the presence of 40% dimethyl sulfoxide. However, for the Na^+/K^+-ATPase the concentration of organic solvent must be raised to 60% in order to block the transfer. At present, we do not know the reason for this difference. It may be that excess solvent, in addition to decreasing the entry of water into the catalytic site after the conversion of E_2-P into $E_1 \sim P$ may also stabilize the E_2-P form and thus impair its conversion into $E_1 \sim P$. This secondary effect might be more pronounced in the case of the Ca^{2+}-ATPase than for the Na^+/K^+-ATPase.

VI. Inorganic pyrophosphatase

There are two different forms of inorganic pyrophosphatase. One of them is a soluble enzyme found in the cytosol of most cells, and the other is found in the chromatophore membranes of photosynthetic bacteria such as *Rhodospirullum rubrum* [106]. The membrane-bound pyrophosphatase can catalyze the synthesis of pyrophosphate in the light. Synthesis is driven by the energy derived from the electrochemical H^+ gradient generated across the membrane of the chromatophore during illumination [106–108]. The comparative study of these two different enzymes has provided useful information for the understanding of the mechanism of energy transduction in biological membranes.

In 1958, Cohn [109] observed that in addition to the hydrolysis of pyrophosphate, the soluble yeast inorganic pyrophosphatase catalyzes an exchange between P_i and pyrophosphate ($P_i \rightleftharpoons PP_i$ exchange) and between the oxygen of P_i and water oxygen ($HOH \rightleftharpoons P_i$ exchange). Cohn [109] concluded that these exchange reactions could not result from the overall reversal of the hydrolysis of PP_i.

In 1979 a mechanism for ATP synthesis by oxidative phosphorylation was being studied in P. Boyer's laboratory [11–13]. His hypothesis was based on a molecular explanation for the rapid $HOH \rightleftharpoons P_i$ exchange reaction catalyzed by the mitochondrial F_1-ATPase. He proposed that the oxygen exchanges were promoted by the reversible formation of ATP at the catalytic site of the ATPase without release of the bound ATP to the medium. By analogy, it seemed possible that the $HOH \rightleftharpoons P_i$ exchange reaction catalyzed by the soluble yeast inorganic pyrophosphatase might result from a correspondingly reversible formation of enzyme-bound PP_i. Accordingly, Janson et al. [20] and later, Springs et al. [21] observed the spontaneous formation of 'tightly bound' pyrophosphate at the catalytic site of the soluble yeast inorganic pyrophosphatase. The amount of enzyme-bound pyrophosphate formed was equivalent to about 5% of the total number of catalytic sites of the enzyme. The kinetic evidence obtained by these two laboratories indicates that two P_i molecules interact on the enzyme surface to form pyrophosphate, and that the hydrolysis of this pyrophosphate leads to incorporation of the oxygen of P_i into water ($HOH \rightleftharpoons P_i$ exchange). In other words, it appears that during the catalytic cycle of the enzyme, there are steps in which the PP_i hydrolyzed is resynthesized on the enzyme surface using the P_i available in the medium. Based on these data, Cooperman and colleagues [21,22] proposed the following reaction sequence:

$$MgPP_i + Mg_2E \underset{(1)}{\rightleftharpoons} Mg_2EMgPP_i \overset{HOH}{\underset{(2)}{\rightleftharpoons}} MgE(MgP_i)_2 \underset{(3)}{\rightleftharpoons} Mg_2E + MgP_i + P_i$$

According to this sequence, reversal of reactions 2 and 3 would account for the $HOH \rightleftharpoons P_i$ exchange, and reversal of reactions 3, 2 and 1 for the $P_i \rightleftharpoons PP_i$ exchange. During $P_i \rightleftharpoons PP_i$ exchange several PP_i molecules are hydrolyzed for each PP_i molecule which, after being synthesized from P_i, dissociates from the enzyme surface to the assay medium.

In conditions similar to those found in the cytosol, the K_{obs} for pyrophosphate hydrolysis is about 10^4 [21,22,28–30]. In contrast, the K_{obs} for the enzyme-bound PP_i (reaction 2 above) is about 5 [21,22]. This situation is similar to that observed for the ATP synthases of both mitochondria and chloroplasts. These

enzymes retain a 'tightly bound' ATP which does not dissociate from the enzyme surface and has K_{obs} for hydrolysis of about 0.5 (Table I). The data of Figs. 2–4 and Table II show that addition of organic solvents to the medium promotes a decrease in the K_{obs} for the hydrolysis of soluble pyrophosphate to values similar to, or even lower than, that measured on the enzyme surface. This finding suggests that the difference in energies of hydrolysis between pyrophosphate in solution and that on the enzyme surface could be ascribed to difference properties of bulk water compared to water on the enzyme surface.

More information on a possible role of water in processes of energy transduction was obtained by comparing the reactions catalyzed by the soluble and the membrane-bound inorganic pyrophosphatase. In the dark, the chromatophores of *R. rubrum* catalyze a $P_i \rightleftharpoons PP_i$ exchange similar to the $P_i = ATP$ exchange described for chloroplasts and mitochondria [46,47,110,111]. The hydrolysis of PP_i leads to formation of a proton gradient [112–114], which in turn promotes the synthesis of PP_i [46,47,110,111]. The $P_i \rightleftharpoons PP_i$ exchange is abolished when a proton ionophore is included in the assay medium. Keister and Raveed [46] compared the $P_i \rightleftharpoons PP_i$ exchange catalyzed by the chromatophores in the dark with that catalyzed by the soluble yeast pyrophosphatase. These authors reported that the K_m for P_i measured in the absence of an H^+ gradient with the soluble enzyme was higher than that measured in the presence of a transmembrane H^+ gradient with the use of chromatophores. De Meis et al. [47] observed that the apparent K_m of P_i for the $P_i \rightleftharpoons PP_i$ exchange reaction varies greatly depending on the Mg^{2+} concentration in the medium and that with the use of either the soluble or membrane-bound enzyme the K_m measured in the presence of 0.5 mM Mg^{2+} was higher than 10 mM P_i and could not be measured. In the presence of this low Mg^{2+} concentration, the addition of organic solvents to the medium promoted a large decrease in the K_m for P_i of the soluble yeast pyrophosphatase (Table III). As discussed for the Ca^{2+}-ATPase, the presence of the organic solvent in the medium would facilitate the partitioning of P_i from the assay medium into the catalytic site if this site has a hydrophobic character when the enzyme is in the form that binds P_i.

Besides the $P_i \rightleftharpoons PP_i$ exchange reaction, the membrane-bound pyrophosphatase of *R. rubrum* chromatophores can also catalyze the net synthesis of pyrophosphate from P_i [106–108,112–115]. During illumination, a proton electrochemical gradient is formed across the membrane and this induces a rapid synthesis of pyrophosphate (Fig. 8, left). The amount of pyrophosphate formed increases with time until a plateau is attained. This level of pyrophosphate remains constant until the light is turned off [107,115]. In the dark, the synthesized

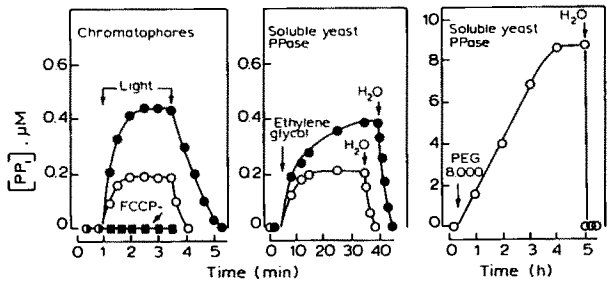

Fig. 8. Synthesis of pyrophosphate by chromatophores of *R. rubrum* and by soluble yeast inorganic pyrophosphatase (PPase). The assay medium composition was 50 mM Tris-HCl buffer (pH 7.8), 10 mM $MgCl_2$ and either 1 mM (○) or 2 mM [^{32}P]P_i (●). Left with chromatophores (total of 0.1 mg bacteriochlorophyll per ml) in the absence (○, ●) and the presence (■) of 5 μM of the H^+ ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The arrows indicate when the light was turned on and off (20 W/m^2). Middle and right with soluble yeast inorganic pyrophosphatase (5 μg/ml). The conditions were as in the left panel except that in the right panel the $MgCl_2$ concentration was 0.9 mM and synthesis of PP_i was measured using 1 mM [^{32}P]P_i. Arrows indicate the addition of organic solvent (↓) and a 20-fold dilution of the added solvent with a solution containing the same buffer, $MgCl_2$, [^{32}P]P_i and enzyme concentration used in the assay (↓H_2O). In the middle panel the solvent added was ethylene glycol, to a final concentration of 60% (v/v) and in the right it was poly(ethylene glycol) 8000 to a final concentration of 50% (w/v). For details see Refs. 31 and 115.

pyrophosphate is rapidly hydrolyzed. Synthesis is abolished when a proton ionophore is included in the assay medium (Fig. 8, left). Recently [115], it was observed that the same profile of variations in pH and the concentration of P_i and $MgCl_2$ that affects the steady-state level of pyrophosphate synthesis measured with chromatophores also affects the level of synthesis attained at equilibrium with soluble yeast inorganic pyrophosphatase, in water or in the presence of organic solvents. With the soluble enzyme, what varies when organic solvent is used, is the total amount of pyrophosphatase synthesized at equilibrium and at each pH, P_i and $MgCl_2$ concentration (Figs. 2–4). This similarity in concentration dependencies of the soluble and membrane bound enzymes suggests that formation of a proton gradient across the chromatophore membrane somehow causes a decrease in the K_{eq} for the hydrolysis of pyrophosphate which allows the accumulation of pyrophosphate in the medium. In fact, Fig. 8 shows that when the water activity of the medium is reduced by the addition of organic solvents, the soluble yeast pyrophosphatase is able to synthesize pyrophosphate in amounts similar to (Fig. 8, middle) or significantly higher than (Fig. 8, right) those synthesized by the chromatophores in the light. With the soluble enzyme; formation of a proton gradient is not feasible.

The data described invite speculation about how a change in hydrophobicity might occur around the membrane bound enzyme. It might be that in chromatophores the catalytic site of pyrophosphatase is situated in the border region between the hydrophilic and hydro-

phobic portions of the membrane. During illumination of chromatophores this part of the enzyme may sink a few nanometers into the hydrophobic region of the membrane. In such a hydrophobic environment the synthesis of pyrophosphate could occur spontaneously, as it does when the water activity of the medium is reduced by the addition of organic solvents (Figs. 2–4 and Table II). The synthesized pyrophosphate could then diffuse from the hydrophobic environment to the hydrophilic medium where it is accumulated until the concentrations of pyrophosphate in the two phases are equilibrated. When the light is turned off the pyrophosphate synthesized during illumination is cleaved because the membrane-bound pyrophosphate regains its hydrophilic location in the membrane. Pyrophosphate would then be of 'low energy' in the hydrophobic phase of the membrane, where the K_{eq} of pyrophosphate hydrolysis is low, and of 'high energy' in the hydrophilic external phase, where the K_{eq} is high. According to this reasoning the energy derived from the proton gradient would be used to induce a conformational change which allows the enzyme to enter into an hydrophobic environment such us that provided by the membrane. This type of movement has been described for the sarcoplasmic reticulum ATPase [116–118]. Mustacich and Weber [119,120] measured the ligand-induced partitioning of different proteins from a water phase into an organic phase as a model to study the partitioning of membrane proteins undergoing a conformational change into or out of the membrane phase. They observed that a small change in the balance between hydrophilic and hydrophobic residues can promote a large change in the partition coefficient of the protein between the two phases and that a potential difference applied across the water/organic solvent interface facilitates the partitioning of the protein. The potential difference in these experiments was similar to that generated across the mitochondrial membrane during respiration.

VII. ATP synthase

The ATP synthase complex of mitochondria, chloroplasts and bacteria catalyzes the synthesis of ATP from ADP and P_i using the energy of the electrochemical proton gradient derived from electron transport. The mechanism by which these enzymes use the energy derived from the gradient is not known. Several theoretical models have been presented in early reports, including, among them, one evoking a possible role for water in the mechanism of ATP synthesis [121–125]. That model, however, was conceptually different from the one reviewed presently. According to the model proposed by Williams [122,123,125], the synthesis of ATP would be driven by a large decrease of water concentration at the catalytic site and the K_{eq} for the hydrolysis of ATP would be high and constant throughout the catalytic cycle of the enzyme. According to our model, based on the data available, the K_{eq} for hydrolysis of the phosphate compound varies greatly during the catalytic cycle in a process involving a rather small change in the water activity at the catalytic site. What follows is a short account of data recently published indicating that perhaps in this system solvation energy may play a role in the process of energy transduction similar to that described for the Ca^{2+}-ATPase. Excellent reviews have been published discussing in detail the many data available on the F_1-F_0 complex [126–132].

Boyer and colleagues observed that soluble F_1 catalyzes an exchange between water oxygen and the oxygen of phosphate [11–13]. These studies indicate that the energy of the proton gradient is not necessary for the synthesis of ATP at the catalytic site of the enzyme but, instead, serves to increase the enzyme affinity for P_i and to permit the dissociation from the enzyme of the tightly bound ATP which forms spontaneously. On the basis of kinetic data, values of K_{obs} for the hydrolysis of tightly bound ATP varying between 2.0 and 0.5 have been reported in different laboratories for both the CF_1 chloroplast ATPase and the F_1 of beef heart mitochondria (Table I). In spite of these low K_{obs} values, several authors [16,17,41–44] failed to detect the spontaneous synthesis of significant amounts of ATP when the mitochondrial F_1 was incubated in a totally aqueous medium containing ADP and a high P_i concentration (10–500 mM). This has been attributed to requirement for the electrochemical proton gradient in raising the apparent affinity of the enzyme for P_i. In the absence of a gradient (soluble F_1), the enzyme affinity for P_i would be so low that the amount of $F_1 \cdot P_i$ complex formed, and thus the amount of tightly bound ATP synthesized, would not be measurable. The proton gradient, in addition to permitting the dissociation of the tightly bound ATP, would also promote a large decrease in the apparent K_m for P_i.

Recently [16,17,41–44], it has been shown that similar to the Ca^{2+}-ATPase, the affinity of soluble mitochondrial F_1-ATPase for P_i increases by several orders of magnitude when part of the water of the incubation medium is replaced by an organic solvent (Table III). Sakamoto [42] reported that the concentration of P_i required for half-maximal synthesis of tightly bound ATP by soluble F_1 is greater than 400 mM in totally aqueous medium and decreases to 0.5 mM in the presence of 40% (v/v) dimethyl sulfoxide (Fig. 9). Similar results were obtained in different laboratories with the use of both mitochondrial [16,17,41,42,44] and bacterial F_1 [43] and with the use of different organic solvents [16]. Organic solvent seems to promote only a decrease on the apparent K_m for P_i, having no effect on the K_{obs} of the tightly bound ATP, which has a value of about one both in the absence and in the presence of

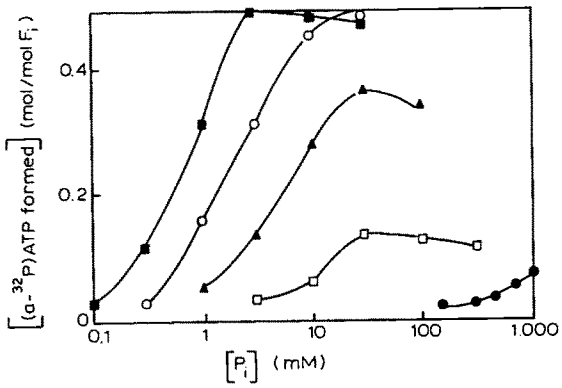

Fig. 9. P_i dependence for the synthesis of 'tightly bound' ATP in the absence and presence of various dimethyl sulfoxide concentrations. F_1 at 6.2 μM was allowed to react for 30 min at 30 °C with 30 μM [γ-^{32}P]ADP and various concentrations of P_i in the presence of 5 mM $MgCl_2$, 0.8 mM EDTA, 11% (w/v) glycerol and 1 mM Mes-Tris buffer (pH 6.8). The dimethyl sulfoxide concentrations were: ●, none; □, 10%; ▲, 20%; ○, 30%; and ■, 40% (w/v). For details see Ref. 42.

organic solvents [16,17]. The tightly bound ATP is synthesized slowly, the rate constant varying between 10 and 5 h^{-1} (Fig. 10). After apparent equilibrium is reached, the P_i which is used for the synthesis of ATP at the catalytic site exchanges with the P_i in the assay medium very slowly, the rate constant of exchange between bound ATP and medium P_i being 0.7 h^{-1} (Fig. 10). In the catalytic site, the rate of exchange between bound ATP and bound P_i occurs more rapidly than the exchange between medium P_i and bound P_i. From $HOH \rightleftharpoons P_i$ exchange experiments, Kandpal et al. [17] estimate that each P_i that binds to the enzyme reversibly forms ATP about 50 times before being released to the

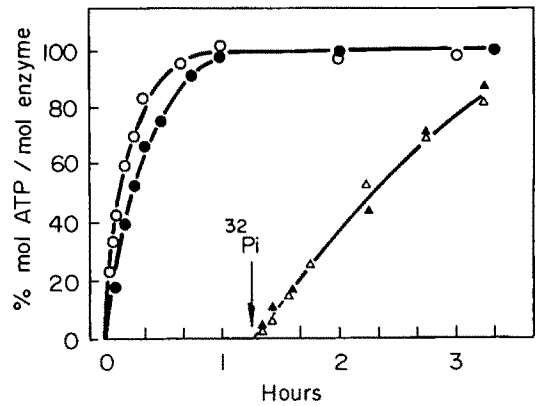

Fig. 10. Exchange of medium P_i with ATP bound to F_1 and to an F_1 preparation containing the inhibitory peptide (F_1-IP). The assay medium contained 50 mM Mes-Tris buffer (pH 6.7), 10 mM $MgCl_2$, 2 mM P_i, 50% dimethyl sulfoxide (v/v) and either 1.2 mg/ml F_1 (○, △) or 0.9 mg/ml F_1-IP (●, ▲). In (○, ●) the enzymes were added to mixtures containing [^{32}P]P_i; samples were withdrawn at the indicated times for assay of bound [γ-^{32}P]ATP. △, ▲, The enzymes were added to mixtures that contained non-radioactive P_i; after 75 min (arrow) carrier-free [^{32}P]P_i was added and samples were withdrawn at the indicated times for assay of bound [γ-^{32}P]ATP. The maximal amounts of ATP synthesized (100%) were 0.36 mol ATP/mol F_1 and 0.41 mol ATP/mol F_1-IP. For details see Ref. 16 (reproduced with permission).

medium. The ATP synthesized from [^{32}P]P_i in the presence of organic solvents remains tightly bound to the enzyme and does not dissociate into the assay medium, even after dilution of the organic solvent or after the addition to the medium of a large excess of either nonradioactive ATP or of a trapping system such as glucose plus hexokinase. The ATPase activity of F_1 is inhibited in the presence of organic solvent concentrations that maximally stimulate the synthesis of ATP. This inhibition is reversible, the ATPase activity being restored after dilution of the organic solvent [16,41,133].

The effect of organic solvents on the apparent K_m for P_i shown in Fig. 9 and the data of Tables I–III indicate that, as proposed for the Ca^{2+}-ATPase, during oxidative phosphorylation the catalytic site of F_1 undergoes a hydrophobic–hydrophilic transition [132]. In the hydrophobic conformation the enzyme binds P_i and, as proposed by Boyer and colleagues [11–13], ATP is synthesized at the catalytic site without the need of energy. Energy would then be needed to promote a conformational change of the enzyme, after which the catalytic site becomes hydrophilic and the bound ATP dissociates from the enzyme.

VIII. Inhibition by hydrophobic molecules

The hydrophobic nature of the active site of both F_1 and Ca^{2+}-ATPase was recently explored by comparing interactions between P_i and different hydrophobic drugs, in the absence and presence of organic solvents [134–136]. Hydrophobic molecules are more soluble in organic solvents than in water. In a system formed by a hydrophilic and a hydrophobic phase, these molecules will be forced out of the hydrophilic into the hydrophobic medium. Thus it would be expected that hydrophobic molecules would compete with P_i and inhibit activity of enzymes involved in energy transduction because they partition from the assay medium (hydrophilic) into the hydrophobic environment of the catalytic site (Fig. 11). The apparent affinity of the enzyme for hydrophobic molecules should decrease when organic solvents are added to the assay medium. The solvent would decrease the difference in hydrophobicity between the active site and the assay medium and thus decrease the force that drives the hydrophobic molecule out of a hydrophilic medium into a hydrophobic environment. This hypothesis was tested using Fe · bathophenanthroline complex and the anti-calmodulin drugs calmidazolium, trifluoperazine and compound 48/80 [134–136]. These molecules are poorly soluble in water and freely soluble in organic solvents. Carlsson and Ernster [137] reported that the Fe · bathophenanthroline complex is a potent inhibitor of F_1-ATPase and of other enzymes involved in energy transduction. Recently [135,136], it was observed that, in addition to inhibition by Fe · bathophenanthroline,

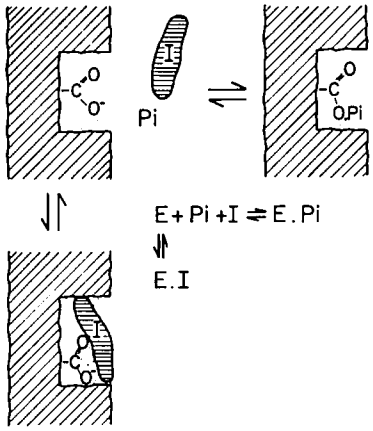

Fig. 11. Inhibition by hydrophobic molecules. The figure shows a schematic representation of the catalytic site of the sarcoplasmic reticulum Ca^{2+}-ATPase. For the synthesis of ATP, the first step of the catalytic cycle of this enzyme is the phosphorylation by P_i of an aspartic acid residue in the catalytic site, forming an acyl phosphate residue. In the figure, I is a hydrophobic molecule which may partition into the hydrophobic environment of the catalytic site and obstruct the entry of the hydrophilic phosphate ion. (Reproduced from Ref. 136 with permission.)

F_1-ATPase activity is also inhibited by different anticalmodulin drugs. Inhibition by these drugs can be reversed either by raising the P_i concentration (Fig. 12) or by adding organic solvents (dimethyl sulfoxide, ethylene glycol or methanol) to the medium [136].

The Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum is not modulated by calmodulin but both ATPase activity and Ca^{2+} uptake as well as phosphorylation by P_i, the fast Ca^{2+} efflux and the synthesis of ATP observed during reversal of the Ca^{2+} pump are inhibited by anticalmodulin drugs [134–136]. The inhibition is reversed by raising the concentration of P_i or by adding dimethyl sulfoxide to the medium. Both Fe · bathophenanthroline and the different anticalmodulin drugs appear to compete with P_i for a common binding site on the Ca^{2+}-ATPase (Fig. 13).

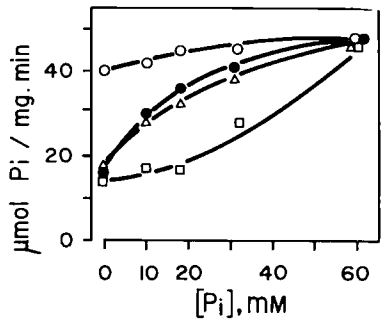

Fig. 12. P_i concentration and inhibition of F_1 by hydrophobic molecules. The assay medium contained 20 mM Mops-Tris buffer (pH 7.0), 3 mM $MgCl_2$, 3 mM ATP and 10 μg/ml soluble F_1 protein. ○, Without inhibitor; □, 2 μM Fe·bathophenanthroline complex; ▲, 12 μM calmidazolium; and ●, 32 μM trifluoperazine. For details, see Ref. 136 (reproduced with permission).

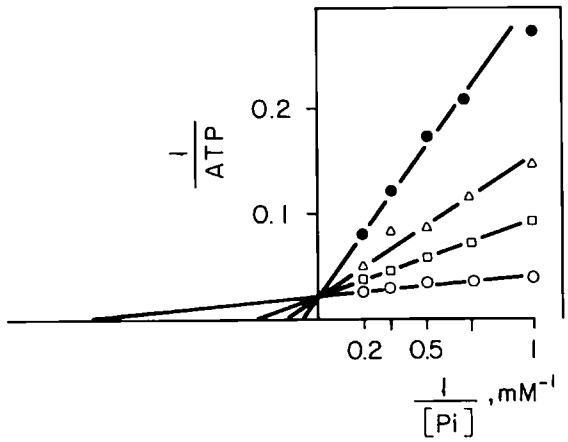

Fig. 13. P_i concentration and inhibition by hydrophobic molecules of ATP synthesis catalyzed by the Ca^{2+}-ATPase. The assay medium contained 50 mM Mops-Tris buffer (pH 6.5), 10 mM $MgCl_2$, 10 mM EGTA, 0.2 mM ADP and the concentrations of P_i shown in the figure. The reaction was started by the addition of vesicles loaded with calcium oxalate (75 μg protein/ml) and quenched after 2 min incubation at 35° C. ○, Control without additions; □, 30 μM trifluoperazine; △, 18 μM Fe·bathophenanthroline complex; ●, 18 μM calmidazolium. For details, see Refs. 134 and 136 (reproduced from Ref. 136 with permission).

IX. Influence of water activity on the hydrolysis rates of acyl phosphate and phosphoanhydride bonds

The catalytic cycle of both the Ca^{2+}-ATPase and Na^+/K^+-ATPase include different forms of phosphoenzyme. The acyl phosphate residue formed from ATP following the binding of the cation and before its translocation through the membrane (reaction 3 in Fig. 5 and reaction 1 in Fig. 7) is hydrolyzed at a much lower rate than the phosphoenzyme form available after translocation and release of the cation on the other side of the membrane (reaction 6 in Fig. 5 and reaction 3 in Fig. 7). If this were not the case, the hydrolysis of ATP would not be coupled to the translocation of the cation through the membrane. In contrast to these data, the findings discussed above indicate that in the first form ($Ca_2 \cdot E \sim P$ and $Na \cdot E_1 \sim P$), the catalytic site is hydrophilic and the acyl-phosphate residue of the phosphoenzyme has a high energy of hydrolysis. In the second form (Ca_2 *E-P and $Na \cdot E_2$-P), the catalytic site is hydrophobic and the acyl phosphate residue has a low energy of hydrolysis.

An apparent paradox in this proposal is that the phosphate compound is hydrolyzed when the catalytic site is hydrophobic. Because water is one of the reactants for the hydrolytic reaction, it would be expected that a decrease in water activity would impair the cleavage of the phosphate compound and not facilitate it. The mechanism of catalysis of the enzymes listed in Table I is not known. One very simple way of trying to understand it is to study the hydrolysis of phosphate compounds that occurs spontaneously in the absence of

enzymes. In 1961, Di Sabato and Jencks [138] observed that at neutral pH, replacement of water by deuterium oxide or the addition of acetonitrile to the reaction medium (50%) had practically no effect on the rate of spontaneous hydrolysis of acetyl phosphate. Kirby and Varvoglis [139] observed that in alkaline solutions the rate of hydrolysis of 2,4-dinitrophenyl phosphate is accelerated many hundred-fold when 70% of the water of the assay medium is replaced by organic solvents such as dimethyl sulfoxide and dimethylformamide. Similar results were recently obtained with the use of acetyl phosphate in alkaline solutions [140]. Substitution of 70% of the water of the medium by dimethyl sulfoxide promoted a two-orders-of-magnitude increase in the rate constant of acetyl phosphate hydrolysis (Fig. 14A). In the presence of 90% dimethyl sulfoxide the rate of hydrolysis was so fast that it could not be measured with the method used. The rate of ATP hydrolysis is also accelerated by organic solvents, both in acid and alkaline solutions (Figs. 14B and C). However, in this case the effect of organic solvents is much less pronounced than that observed for acetyl phosphate hydrolysis. Acceleration of both acetyl phosphate and ATP hydrolysis is promoted by a decrease in both activation energies (E_a) and in entropies of activation (Fig. 15).

Extrapolated to the Ca^{2+}-ATPase and to the Na^+/K^+-ATPase, these data suggest that a decrease in water activity in the catalytic site probably does not impair the hydrolysis of the acyl phosphate residue; on the contrary, it may promote a decrease in the activation energy (E_a) of hydrolysis. The same reasoning might also apply to the F_1, which spontaneously forms tightly bound ATP from ADP and P_i. In this enzyme, two forms of enzyme-substrate complex are formed in sequence during the catalytic cycle. In one of them ATP has a high energy of hydrolysis, dissociates easily from the enzyme and is hydrolyzed at a low rate. In the second, ATP has a low energy of hydrolysis, does not dissociate from the enzyme and is hydrolyzed rapidly.

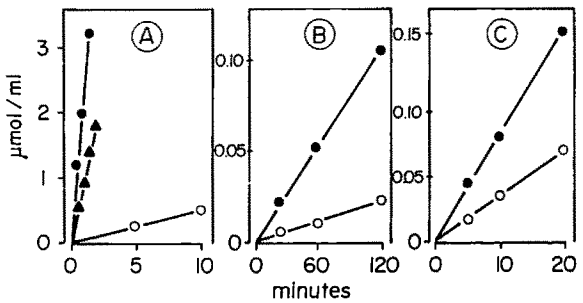

Fig. 14. Spontaneous hydrolysis of acetyl phosphate and of ATP. In (A) the media contained 0.1 M NaOH, 5 mM acetyl phosphate and either no further addition (○) or 50% (v/v) of either ethylene glycol (▲) or Me_2SO (●). The temperature was 25°C. In (B) the media contained 1 mM NaOH, 10 mM $MgCl_2$, 1 mM ATP and either no further addition (○) or 50% dimethyl sulfoxide (●). The temperature was 30°C. In (C) the media contained 1 M HCl, 1 mM ATP and either no further addition (○) or (●) 50% (v/v) dimethyl sulfoxide. The temperature was 35°C. For details see Ref. 140.

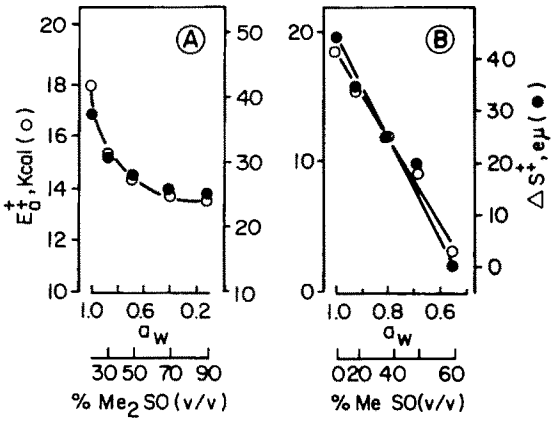

Fig. 15. Activation energies and entropies of activation for the hydrolysis of acetyl phosphate (A) and of ATP in media with different water activities. In (A) hydrolysis was measured at 25, 30, 35 and 40°C in media containing 0.1 M NaOH, 5 mM acetyl phosphate, 10 mM $MgCl_2$ and different concentrations of dimethyl sulfoxide. Activation energies (○) and entropies of activation (●) were calculated from the Arrhenius plot of the rate constants measured at different temperatures. In (B) the medium contained 1 M HCl, 1 mM ATP and the concentrations of dimethyl sulfoxide shown in the figure. Rates of hydrolysis were measured at 30, 35, 40, 45 and 50°C. The water activity (a_w) corresponding to the different dimethyl sulfoxide (Me_2SO) concentrations used were determined from vapor pressure values [60]. For details, see Ref. 140.

At present we do not know why a decrease in water activity promotes a decrease in both E_a and $\Delta S^{\ddagger}$. The water molecules which organize around the acyl phosphate residue of acetyl phosphate [23,25] and the phosphoanhydride bond of the γ-phosphate of ATP (Table IV) have a low mobility and are strongly held in a position which depends on the charge distribution around the molecule [23,25,52]. Because of their low mobility, these water molecules may not participate in the hydrolytic reaction; in addition, their presence may impair the access of other water molecules and/or of H^+ and OH^- ions to the region of the phosphate compound where the bond would be cleaved, thus impeding completion of the hydrolytic reaction. It may be that the addition of organic solvents promotes a change in the solvation shell of the phosphate compound which facilitates access to the bond by either OH^- or water molecules with higher mobility, thus favoring the hydrolytic reaction.

X. Conclusions

The data obtained with the Ca^{2+}-ATPase, Na^+/K^+-ATPase, F_1 and inorganic pyrophosphatase suggests that solvent structure at the catalytic site is involved in the process of energy transduction. It may be that, for the different membrane-bound enzymes, the energy

derived from ionic gradients is used to create media with a particular composition and structure of the solvent. In one medium (e.g., of low water activity) the phosphate compound would be of low energy and could be formed spontaneously. In a second medium (e.g., of high water activity) the molecule would acquire high-energy characteristics. Media of different compositions can be found in different compartments of the cell, as for instance in the cytosol and in the lumen of a cell organelle or, at the molecular level, in the catalytic site of an enzyme. Energy would be used by the enzyme to change its conformation, and two different microenvironments would be found at the catalytic site: one before and another after the conformational change. According to the mechanism proposed [33,68], synthesis of a compound and its change from low energy to high energy can occur in the presence of a large excess of water because it is not dependent on the water concentration in the medium but on the manner in which water is organized around the molecule. Note that in Figs. 2–4, the water concentration in the different mixtures of solvent used varied between 22 and 39 M. Thus, a change in the structure of bulk water may lead to a small change in the solvation shell of reactant and products and this can promote a significant change in the thermodynamic parameters of the reaction.

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