Biochimica et Biophysica Acta, 311 (1973) 272–291

BBA 76357

EFFECTS OF SOME ORGANIC SOLVENTS ON THE REACTIVITY OF SODIUM *PLUS* POTASSIUM ION-TRANSPORT ATPase

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(Received February 5th, 1973)

SUMMARY

1. Organic solvents including aliphatic alcohols, acetone, diethyl ether and chloroform inhibited ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) of the plasma membranes of guinea pig kidney. Inhibition was uncompetitive with respect to ATP if the ATP concentration was larger than 5 μM. Below this concentration of ATP, K^+ inhibits rather than stimulates the Na^+-dependent splitting of ATP by the native enzyme. The tested organic solvents converted this K^+ inhibition into K^+ stimulation. In the presence of these solvents, the apparent affinity of the enzyme increased for Na^+ and ATP and decreased for K^+ as estimated by the kinetics of ATP hydrolysis. Apparent homotropic allosteric interaction between the Na^+ and K^+ sites increased.

2. Organic solvents interfered with different steps of the transient phosphorylation of the enzyme. As estimated by pulse labeling, organic solvents enhanced interaction of ATP with the enzyme prior to phosphorylation both in the absence or in the presence of K^+. The solvents decreased the rate of dephosphorylation of the phospho-enzyme either in the presence of K^+ (native enzyme) or in the presence of ADP (*N*-ethylmaleimide-treated enzyme) but they did not affect the ratio of the ADP-sensitive form of the native phospho-enzyme to the K^+-sensitive form. Rephosphorylation by ATP of the dephospho-enzyme, formed by adding Rb^+, was accelerated by organic solvents.

3. Interaction of the enzyme with these organic solvents appeared to be primarily hydrophobic, as the half-maximal inhibitory concentrations of these solvents correlated with their octanol–water partition coefficients and with the length of the hydrophobic side chain in the series of homologous aliphatic alcohols.

4. It is considered that these modifications of ($Na^+ + K^+$)-ATPase were due partly to conformational changes of the enzyme, and partly to changes in the water structure around the active center which is proposed to be in a hydrophobic environment.

Abbreviation: CDTA, (1,2-cyclohexylenedinitrilo) tetraacetic acid.

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INTRODUCTION

$(Na^+ + K^+)$-ATPase (EC 3.6.1.3) is an enzymatic manifestation of the active, linked transport of Na^+ and K^+ across plasma membranes[1]. Ethanol inhibits both the transport process[2] and the enzyme[3–5]. Lower aliphatic alcohols, acetone, chloroform and diethyl ether can also produce this inhibition which is more prominent at low concentrations of K^+. The reaction sequence of $(Na^+ + K^+)$-ATPase involves the formation and breakdown of a phospho-enzyme[6]. Ethanol did not affect phosphorylation but appeared to inhibit primarily the dephosphorylation step[7]. These data suggest that organic solvents may be selective inhibitors of some of the partial reactions of $(Na^+ + K^+)$-ATPase and thus help to elucidate the reaction mechanism of the enzyme.

This paper is a study of the effects and of the mechanism of action of some organic solvents on $(Na^+ + K^+)$-ATPase. The solvents studied increased the affinity of the enzyme for ATP and Na^+ but decreased its affinity for K^+. With respect to dephosphorylation, organic solvents stabilized the phosphorylated native enzyme in the presence of K^+ and the phosphorylated, *N*-ethylmaleimide-treated enzyme in the presence of ADP, but, they did not affect the ratio of ADP-sensitive form of the phospho-enzyme to K^+-sensitive form. The splitting of ATP by the enzyme decreased because of this inhibited dephosphorylation. The effectiveness of organic solvents correlated with their hydrophobicity.

MATERIALS AND METHODS

Materials

The following organic solvents were of "spectrograde" quality: acetone *N,N*-dimethylformamide, *n*-propanol, isopropanol, *n*-butanol and isobutanol. The other organic solvents were of "A.C.S. certified" quality, and were used without further purification. [^{32}P]ATP was synthesized enzymatically[8], membranes containing $(Na^+ + K^+)$-ATPase from guinea pig kidney cortex were prepared and treated with NaI as described earlier[8,9].

Assay procedures

$(Na^+ + K^+)$-ATPase activity was estimated in duplicate samples by two different methods. At ATP concentrations larger than 0.1 mM, the amount of inorganic phosphate liberated from ATP was measured by an automated procedure on the Technicon Autoanalyzer as described previously[9] (Method A). In 1 ml, the reaction medium for total ATPase activity contained: 100 μmoles of NaCl, 25 μmoles of KCl, 4 μmoles of $MgCl_2$, 4 μmoles of Tris–ATP (pH 7.4), 30 μmoles of imidazole glycylglycine (pH 7.4) and 10 to 30 μg of membrane protein. An otherwise similar medium contained 0.25 μmole of ouabain in place of the monovalent inorganic cations in order to estimate non-specific ATPase activity. $(Na^+ + K^+)$-ATPase activity was the difference between two activities. If the ATP concentration was lower than 0.1 mM, a different procedure was used (Method B). The reaction mixture contained in 1 ml; 0.1–50 nmoles of [^{32}P]ATP, 0.5–1.0 μmole of $MgCl_2$, 10 μmoles of imidazole–HCl (pH 7.4), 10–30 μg of membrane protein and various amounts of NaCl, KCl and organic solvent. Non-specific ATPase activity was estimated by replacing Na^+ and K^+ with 0.25 μmole of ouabain in the reaction mixture. 0.9 ml of the reaction mixture,

with the omission of the [^{32}P]ATP, was prewarmed at 37 °C for 4 min. The reaction was started by the addition of [^{32}P]ATP, in 0.1 ml and was terminated after 30 s by adding 0.5 ml of 1.2 M $HClO_4$ containing 1 mM H_3PO_4. Inorganic phosphate was extracted into butyl acetate as phosphomolybdate complex by the method of Wahler and Wollenberger[10]. Radioactivity of an aliquot of this butyl acetate extract was measured in a gas flow thin-window counter. One unit of enzyme splits 1 μmole of ATP per min at 37 °C.

For the estimation of K^+-*p*-nitrophenylphosphatase at 37 °C, the reaction mixture contained in 1 ml: 3 μmoles of *p*-nitrophenyl phosphate, 3 μmoles of $MgCl_2$, 10 μmoles of imidazole glycylglycine (pH 7.4), 20–50 μg of membrane protein and various amounts of KCl, NaCl and Tris–ATP. Non-specific activity was estimated by adding 0.25 μmole of ouabain to the basic medium while omitting inorganic monovalent cations. After 20 min, the reaction was stopped by the addition of 2 ml of 0.1 M NaOH and the amount of *p*-nitrophenol liberated was measured on a spectrophotometer at 400 nm. One unit of enzyme splits one μmole of *p*-nitrophenyl phosphate per min at 37 °C.

Protein was estimated by Miller's modification[11] of the procedure of Lowry *et al.*[12].

Membranes were phosphorylated by the method of Post and Sen[8], and the amount of the phospho-enzyme was estimated by Millipore filtration as described previously[13].

RESULTS

Three features were common for all organic solvents used in these experiments. First: below a certain concentration the effects of these solvents were reversible, at higher concentrations the solvents denatured the enzyme irreversibly. This paper reports only the reversible effects of the solvents. The inhibition was reversible at these low concentrations because the effects of a solvent were abolished if the solvent was washed off the membranes by repeated centrifugation and resuspension. Therefore, when an organic solvent was used it was added directly to the reaction mixture. Second: none of the organic solvents was specific in producing any of the phenomena described here. Acetone, ethanol and *n*-butanol were used most often for technical convenience and experiments done with these solvents are most often referred to for easier comparison. Third: the effects of any of these solvents were apparently independent of time since exposure of the enzyme to a solvent for 5 s or for 45 min produced the same changes.

$(Na^+ + K^+)$-ATPase required Na^+ and K^+ simultaneously for maximal activation if the concentration of ATP is larger than about 5 μM[1]. This is an enzymatic manifestation of the tight coupling of Na^+ and K^+ transport. At ATP concentration below 5 μM, K^+ inhibits the Na^+-dependent splitting of ATP by the enzyme[13,14]. Organic solvents affected the kinetics of ATP splitting differently in these two ranges of ATP concentration.

Effects of organic solvents on the splitting of ATP by the enzyme at ATP concentrations above 5 μM

In these experiments, the following organic solvents were used: aliphatic

alcohols from methanol to *n*-decanol, acetone, chloroform and diethyl ether. Low concentrations of these solvents slightly (20%–30%) but consistently activated $(Na^+ + K^+)$-ATPase (not shown). This activation probably resulted from a better dispersion of the crude membrane preparation as shown by a decrease in the turbidity of the suspension.

Higher concentrations of the solvents inhibited the enzyme. Within the range of reversible inhibition, these solvents inhibited $(Na^+ + K^+)$-ATPase uncompetitively with respect to ATP, *i.e.* the inhibitor decreased V but increased the apparent affinity for ATP (refs 15, 16). Uncompetitive inhibition is characterized by parallel lines in a double reciprocal, Lineweaver–Burk plot (Panel A of Fig. 1). The slopes of these straight lines were 0.34 in the absence and 0.33 in the presence of acetone, as calculated by the least squares method, indicating that the two lines were practically parallel. The apparent K_m for ATP was 0.8 mM for the native enzyme and 0.34 mM in the presence of acetone. As the accuracy of determining these slopes in double reciprocal plots is technically limited, these and similar data were replotted according to Hunter and Downs[17] (Panel B of Fig. 1). This plot is particularly suitable for establishing the uncompetitive nature of an inhibition, as in this plot competitive and non-competitive inhibitions give straight lines; however, uncompetitive inhibition gives a hyperbolic curve, as in the present case. (Note that in Fig. 1 the concentration of ATP was kept above 0.1 mM. At lower ATP concentrations, particularly below 5 μM, organic solvents activated the enzyme in the presence of both Na^+ and K^+).

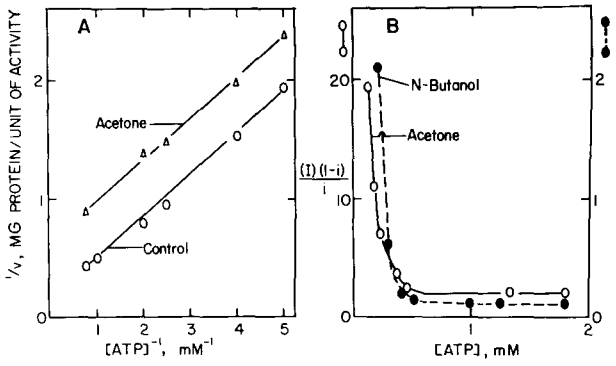

Fig. 1. Inhibition of $(Na^+ + K^+)$-ATPase by acetone and *n*-butanol. Panel A is a double reciprocal Lineweaver–Burk plot. ○—○, control; △—△, with 2.04 M acetone. In Panel B, data were plotted according to Hunter and Downs[17]. In this plot, competitive and non-competitive inhibitions give straight lines; uncompetitive inhibition gives a hyperbolic curve. I is the concentration of the inhibitor (acetone or *n*-butanol) which is kept constant, i is the fraction of enzyme activity inhibited at each ATP concentration. The left ordinate refers to values obtained with acetone, the right ordinate refers to values obtained with *n*-butanol. ○—○, with 2.4 M acetone; ●—●, with 0.216 M *n*-butanol. The enzyme activity was estimated by Method A.

The half-maximal inhibitory concentrations of various solvents at a saturating concentration of ATP and at 37 °C are shown in Table I.

Besides ATP, $(Na^+ + K^+)$-ATPase has three other ligands: Mg^{2+}, Na^+ and K^+. The effect of Na^+ is unique; K^+ can be replaced by Tl^+, Rb^+, NH_4^+, Cs^+ or Li^+; Mg^{2+} can be replaced by Mn^{2+} (ref. 1). Na^+ and K^+ both have multiple binding sites with homotropic cooperative interaction among the sites[18]. In order to deter-

TABLE I

HALF-MAXIMAL INHIBITORY CONCENTRATIONS OF SOME ORGANIC SOLVENTS

Half-maximal inhibitory concentrations were estimated either from plots of inhibitor concentration *versus* $(Na^+ + K^+)$-ATPase activity, or from plots as in Panel B of Fig. 1. $(Na^+ + K^+)$-ATPase activity was estimated by Method A.

Organic solvent	*Half-maximal inhibitory concn (M)*
Methanol	2.52
Ethanol	1.17
n-Propanol	0.39
Isopropanol	0.62
n-Butanol	0.16
Isobutanol	0.20
n-Pentanol	0.062
n-Hexanol	0.017
n-Heptanol	0.0073
n-Octanol	0.0042
n-Nonanol	0.003
Acetone	1.32
Chloroform	0.023
Diethyl ether	0.29

TABLE II

EFFECT OF THREE ORGANIC SOLVENTS ON THE ACTIVATION OF $(Na^+ + K^+)$-ATPase BY Na^+ AND K^+

At 37 °C in 1 ml the reaction mixture contained 2 μmoles of Tris–ATP (pH 7.5), 2 μmoles of $MgCl_2$ and 10 μmoles of imidazole–HCl (pH 7.4). In order to estimate the kinetic parameters of activation by Na^+ and K^+, the concentration of one of these cations was kept constant at the indicated level ("fixed cation") while the concentration of the other cation was varied. Apparent half-maximal activating concentrations ($K_{0.5}$) and apparent interaction coefficients (Hill coefficient, *n*) of multiple sites were calculated from Hill plots. $(Na^+ + K^+)$-ATPase was assayed by Method A. These concentrations of the three solvents reduced *V* by 50%.

Organic solvents	*Concn (M)*	*Fixed cation*		$K_{0.5}$		*Hill coefficient (n)*
		Na^+ (mM)	*K^+ (mM)*	*Na^+ (mM)*	*K^+ (mM)*	
None	—	100	—	—	0.47	0.9
		—	25	18.6	—	1.1
Acetone	1.32	100	—	—	2.18	1.3
		—	25	10.2	—	1.3
Ethanol	1.17	100	—	—	2.20	1.3
		—	25	6.5	—	1.3
n-Butanol	0.16	100	—	—	2.70	1.3
		—	25	5.8	—	1.2

mine kinetic parameters of activation by Na^+ and K^+, it was convenient to keep one of these ions at a constant level ("fixed cation" in Table II) and to vary the concentration of the other ion. ATP and Mg^{2+} were equimolar and saturating. Acetone, ethanol and *n*-butanol were present in half-maximal inhibitory concentration (*cf.* Table I). These three solvents increased the apparent affinity (*i.e.* decreased $K_{0.5}$) of the enzyme for Na^+ by a factor of 3; in contrast they decreased the apparent affinity for K^+ by a factor of 10 (Table II). (The symbol $K_{0.5}$ refers to the concentration of a ligand at half-maximal velocity of the enzyme.) Apparent cooperativity among Na^+ and K^+ sites also increased as indicated by a larger Hill coefficient. This increase was small but statistically significant. At their half-maximal inhibitory concentrations, these three solvents did not bring about the same quantitative changes in the kinetic parameters but the direction of these changes was similar. Double reciprocal plots of these data failed to indicate simple competitive antagonism between K^+ and any of the three solvents. As the concentration of the added solvent rose there was a continuous deviation from linearity in the double reciprocal plots (not shown) which made inaccurate the estimation of the slopes of the lines and the estimation of their intercepts by extrapolation.

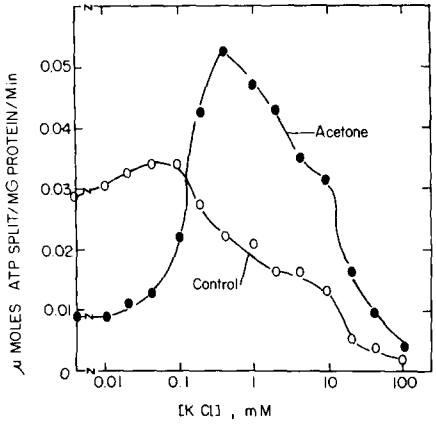

Fig. 2. Effect of K^+ on the splitting of ATP at a low concentration of ATP. 1 ml of the reaction medium contained 0.8 nmole of [^{32}P]ATP, 1 μmole of $MgCl_2$, 16 μmoles of NaCl, 0.115 mg of membrane protein and 5 μmoles of imidazole–HCl (pH 7.5). The concentration of KCl is shown on the abscissa. ○—○, control; ●—●, with 1.32 M acetone. Non-specific ATPase was estimated by adding 0.25 μmole of ouabain in place of inorganic monovalent cations both in the absence or in the presence of 1.32 M acetone. The non-specific activity was subtracted from the corresponding total activity and this difference is plotted on the graph. Incubation was at 37 °C for 30 s. The liberated ^{32}P-labeled P_i was estimated by Method B. (The activity of the enzyme at 4 mM ATP was 1.53 units/mg protein, as estimated by Method A.)

In contrast, Israel reported simple competitive antagonism between ethanol and K^+, but he did not observe interaction among K^+ sites probably because he studied a narrower range of K^+ concentrations[5].

Reversal by organic solvents of K^+ inhibition observed below 5 μM ATP

At any concentration of ATP, Na^+ stimulates the splitting of ATP by the enzyme. The effect of K^+ on this Na^+-dependent splitting of ATP is modified by

the concentration of ATP. If the concentration of ATP is high, (close to saturation) K^+ activates the enzyme and K^+ suppresses the splitting of ATP below the Na^+-dependent level only, if the K^+ concentration is higher than the Na^+ concentration. At low concentrations of ATP, particularly below 5 μM, only low concentrations of K^+ activated the enzyme (less than 0.3 mM at 0.8 μM ATP, Fig. 2); higher concentrations of K^+ inhibited the Na^+-dependent splitting of ATP. Thus, below about 5 μM ATP, inhibition by K^+ was predominant over activation by K^+. This phenomenon was interpreted as indication of a separate enzyme (called "Na^+-dependent ATPase")[14,19,20], or inhibition of ($Na^+ + K^+$)-ATPase[13,21,22]. At 0.8 μM ATP acetone affected the splitting of ATP in three different ways (Fig. 2). First: acetone inhibited two-thirds of the Na^+-dependent activity. Second: acetone converted inhibition by K^+ into activation by K^+, except at very high K^+ concentrations. Quantitatively: in the absence of acetone 0.06 mM K^+ activated the enzyme maximally and inhibition of the Na^+-dependent activity began at 0.3 mM K^+; in the presence of 1.32 M acetone 0.6 mM K^+ activated the enzyme maximally and inhibition of the Na^+-dependent activity began at 60 mM (the concentration of Na^+ was 16 mM in these experiments). Third: acetone increased by 60% the maximal activity of the enzyme at optimal concentration of Na^+ and K^+. The transformation of K^+ sensitivity was a function of acetone concentration (Table III). With increasing acetone level the Na^+-dependent activity decreased to practically zero, however, the ($Na^+ + K^+$)-dependent activity increased. These phenomena were reproduced with acetone, ethanol, *n*-butanol, isobutanol, diethyl ether and *N,N*-dimethylformamide.

TABLE III

EFFECT OF ACETONE ON ATPase ACTIVITY AT A LOW CONCENTRATION OF ATP

1 ml of the reaction mixture contained: 2 nmoles of [^{32}P]ATP, 1 μmole of $MgCl_2$, 5 μmoles of imidazole–HCl (pH 7.5), 10 μg of membrane protein, NaCl, KCl and acetone as indicated. ATPase activity was assayed and corrected for non-specific splitting by Method B.

Concn of acetone (M)	*ATPase activity (unit/mg protein) in the presence of*	
	NaCl (50 mM)	*NaCl (50 mM) + KCl (2 mM)*
0.0	0.041	0.024
0.4	0.021	0.043
0.8	0.011	0.059
1.3	0.005	0.065
1.6	0.004	0.068

Effects of some solvents on the transient kinetics of the phospho-enzyme

The splitting of ATP by ($Na^+ + K^+$)-ATPase involves transient phosphorylation of the enzyme[1]. The first step of the phosphorylation cycle is probably the binding of ATP to the enzyme. K^+ decreases the binding affinity for ATP, Na^+ antagonizes this effect of K^+ (refs 9, 23, 24). In the presence of Na^+ and Mg^{2+} the terminal phosphate of ATP is transferred to the enzyme and forms an acyl-phosphate bond. K_m for ATP in the phosphorylation of the enzyme is less than 1 μM

(ref. 25). The steady-state level of the phospho-enzyme is established in about 1 s even at a low concentration of ATP (10 μM) (ref. 26). The native phospho-enzyme is labile at neutral pH. Dephosphorylation is slow in the presence of Na^+ alone (the rate constant is about 0.06 s^{-1}) but dephosphorylation is accelerated in the presence of K^+ (rate constant is about 4.4 s^{-1} in the presence of 0.6 mM K^+)[26]. A different phospho-enzyme is formed after poisoning the enzyme with *N*-ethylmaleimide[27]. The splitting of this phospho-enzyme (called E_1–P) is not accelerated by K^+, but it can transfer its phosphate reversibly to ADP. In the native enzyme, a form similar to E_1–P is probably a precursor of the K^+-sensitive form (called E_2–P) but these forms may possibly lie on alternative pathways of the reaction sequence[28]. One may imagine that in the intact cell E_2–P combines with K^+ present in the extracellular solution and subsequent splitting of E_2–P may be related to inward translocation of K^+ across the membrane. Intracellularly, the inorganic phosphate appears to dissociate first, leaving behind an enzyme–K^+ complex. This complex must dissociate before the phosphorylation cycle can be initiated again.

Ideally the effects of organic solvents on all these steps should be investigated. The manual pipetting method used in this work was, however, too slow to measure the rate of phosphorylation or the rate of dephosphorylation in the native enzyme if more than 0.06 mM K^+ was present. It was possible, however, to estimate: (a) the binding of ATP to the enzyme; (b) the level of the phospho-enzyme (probably in the steady state); (c) lower rates of dephosphorylation in the native enzyme (if less than 0.06 mM K^+ were added); and (d) the rate of rephosphorylation of the dephospho-enzyme formed after the addition of Rb^+. In the following sections (A–D) the effects of some organic solvents on these steps of the phosphorylation cycle will be presented. Acetone, aliphatic alcohols (from ethanol to *n*-heptanol), diethyl ether and *N*,*N*-dimethylformamide were used in these experiments.

(A) Binding of ATP to the enzyme

$(Na^+ + K^+)$-ATPase contains one high affinity and possibly several low affinity binding sites for the substrate, ATP. Na^+ slightly promotes, K^+ strongly inhibits ATP binding (K^+ decreases binding affinity at least 50-fold), Mg^{2+} is not required[9,23,24]. In previous experiments ATP binding was studied by a flow dialysis method[9]. This method could not be used in the presence of organic solvents as the solvents appeared to change (generally decrease) the diffusion of ATP through the dialysis membrane progressively during the experiment. The binding of ATP was therefore estimated by pulse labeling. This procedure measures that fraction of bound ATP which was precursor of the phospho-enzyme[29].

[^{32}P]ATP was added first to Mg^{2+}-free enzyme. After binding probably took place (10 s) the enzyme was phosphorylated by adding Na^+, Mg^{2+} and a 250-fold excess of unlabeled ATP. Na^+ and Mg^{2+} initiated phosphorylation and the unlabeled ATP immediately diluted the labeled ATP, so that only those enzyme molecules became labeled which had radioactive ATP bound to them before the addition of Na^+ and Mg^{2+}. (The enzyme was not phosphorylated in the absence of Na^+ and Mg^{2+} and in the presence of (1,2-cyclohexylenedinitrilo) tetraacetic acid, CDTA.) But this system is more complicated than simple substrate binding. During labeling the free ATP, the free enzyme, the enzyme–ATP complex and the phospho-enzyme are in the steady state and organic solvents may modify any component or

reaction in this system. Nevertheless, the amount of the phospho-enzyme is proportional to the amount of [^{32}P]ATP bound to the enzyme beforehand. The amount of the phospho-enzyme was estimated by extrapolation of the turnover line to zero time (Fig. 3). In one sample [^{32}P]ATP was bound to the enzyme in the absence of monovalent inorganic cations, in another sample [^{32}P]ATP was bound to the enzyme in the presence of 0.05 mM K^+. K^+ decreased binding by about 90% (open symbols in Fig. 3). If acetone was also present practically the same amount of ATP was bound to the enzyme irrespective of the presence or absence of K^+ (shaded symbols in Fig. 3). Thus, acetone, and in a similar experiment ethanol, prevented the action of K^+ and enhanced ATP binding. It is likely that acetone did dissociate K^+ from the enzyme but the association or dissociation of K^+ was not determined directly.

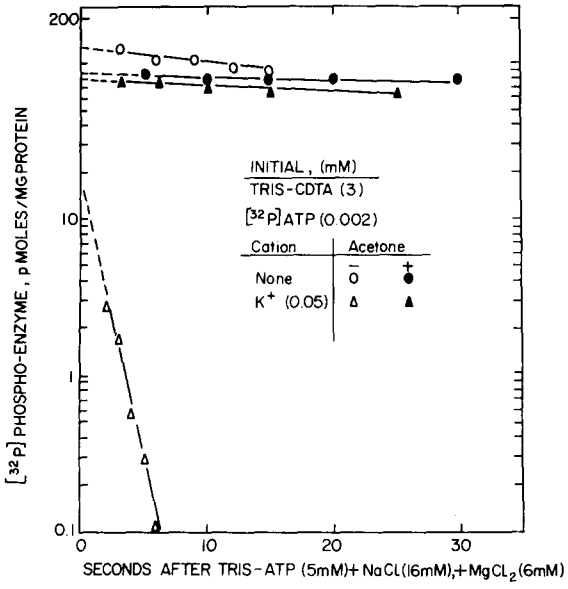

Fig. 3. Effect of acetone on pulse labeling of ($Na^+ + K^+$)-ATPase with [^{32}P]ATP. At 0 °C in 0.8 ml the control samples (○, △) contained: 0.48 mg of membrane protein, 3 μmoles of Tris–CDTA (pH 7.5), 10 μmoles of imidazole–glycylglycine (pH 7.5) without (○) or with (△) 0.05 μmole of KCl. In addition, the experimental samples (●, ▲) contained 0.3 ml of acetone, without (●) or with (▲) 0.05 μmole of KCl. First, 2.3 nmoles of [^{32}P]ATP was added in 0.1 ml. 10 s later (this is zero time on the graph) 5 μmoles of Tris–ATP (pH 7.5), 6 μmoles of $MgCl_2$ and 16 μmoles of NaCl were added in 0.1 ml. The reaction was stopped with acid at the times indicated and the amount of the phospho-enzyme was estimated as described in Methods and Materials. From each value, presented on the graph, the blank phosphorylation of the membranes was subtracted. This blank phosphorylation was estimated by labeling the membranes with [^{32}P]ATP in the presence of 16 mM KCl and 1 mM $MgCl_2$ for 5 s. The slopes of the lines were calculated by the least-squares method.

(B) Phospho-enzyme levels

Phosphorylating capacity of a preparation was estimated by incubating the membranes with [^{32}P]ATP, Na^+ and Mg^{2+} for 5 s. (5 s were probably enough for establishing the steady-state level of the phospho-enzyme[26,30]). The effect of acetone on the phospho-enzyme level depended on the concentration of ATP (Table IV,

Expt A). Progressively increasing concentrations of acetone first increased and later decreased the level of the phospho-enzyme (Table IV). (This decrease coincided with the irreversible inactivation of the enzyme by higher concentrations of acetone.) These experiments were reproduced with acetone, *n*-butanol and isobutanol.

TABLE IV

EFFECT OF ACETONE ON THE LEVEL OF PHOSPHO-ENZYME AT DIFFERENT CONCENTRATIONS OF ATP

At 0 °C in 1 ml, the reaction mixture contained 0.36 mg of membrane protein, 1 μmole of $MgCl_2$, 10 μmoles of imidazole–glycylglycine (pH 7.5), 16 μmoles of NaCl or KCl (but not both) and [^{32}P]ATP as shown. The level of phospho-enzyme is the amount formed in 5 s in the presence of 16 mM NaCl *minus* the amount formed in the presence of 16 mM KCl at the same concentration of ATP. The estimation of the phospho-enzyme is described in Methods and Materials.

Expt	*Concn of acetone (M)*	*Concn of [^{32}P]ATP (μM)*	*Phospho-enzyme (pmoles ^{32}P/mg protein)*
A	none	2	164
	1.32	2	252
	none	40	425
	1.32	40	434
B	none	20	302
	0.87	20	335
	1.74	20	341
	2.71	20	294
	3.48	20	225
	4.35	20	82
	5.22	20	23

(*C*) *Dephosphorylation*

The rate of spontaneous dephosphorylation (in the presence of Na^+ alone) of the phospho-enzyme can be estimated by blocking radioactive rephosphorylation with CDTA which chelates Mg^{2+} or with unlabeled ATP which dilutes the label in the substrate[25]. Dephosphorylation of E_1–*P* can be stimulated and thereby estimated by adding ADP; dephosphorylation of E_2–*P* can be estimated similarly by adding K^+ (or its congeners) with CDTA or with unlabeled ATP. The native enzyme forms primarily E_2–*P*, a small amount of E_1–*P* can be detected in the presence of high concentrations of Na^+ (Post, R. L., personal communication). The *N*-ethylmaleimide-treated enzyme forms exclusively E_1–*P*.

Acetone lowered the rate of spontaneous dephosphorylation 6–7 fold. (Panel A of Fig. 4). The rates of breakdown of the phospho-enzyme were similar either with CDTA or with unlabeled ATP in the blocking solution. Addition of ADP together with unlabeled ATP did not accelerate dephosphorylation indicating that no significant amount of E_1–*P* accumulated neither in the absence nor in the presence of acetone. It is important to note, therefore, that acetone did not obviously inhibit the conversion of E_1–*P* to E_2–*P*. Actually, the rate of dephosphorylation was somewhat slower

if ATP and ADP were added together. This "stabilization" of the phospho-enzyme was not specific for ADP but appeared to be a non-specific effect of high concentration of nucleotides. In this experiment the total concentration of ATP and ADP was 10 mM. In similar experiments, 5–10 mM ATP or ITP also stabilized the phospho-enzyme (not shown).

Acetone also inhibited the K^+-stimulated hydrolysis of E_2–P by a factor of 9. E_2–P, however, did not become entirely insensitive to K^+ since a 12.5-fold increase in the concentration of K^+ restored the original rate of breakdown (Panel B of Fig. 4).

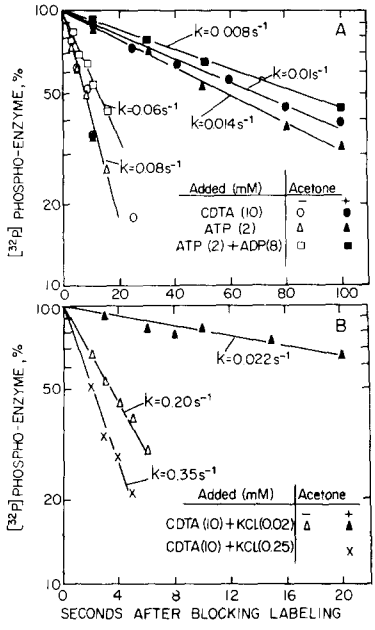

Fig. 4. Effect of acetone on the hydrolysis of the phospho-enzyme. At 0 °C in 0.8 ml, the reaction mixture contained 16 μmoles of NaCl, 1 μmole of $MgCl_2$, 10 μmoles of imidazole glycylglycine (pH 7.5), 0.525 mg of membrane protein and 1.32 mmoles of acetone as indicated. The reaction was started by the addition of 0.04 μmole of [^{32}P]ATP in 0.1 ml. After 5 s, phosphorylation was blocked by the addition in 0.1 ml of: (in Panel A) 10 μmoles of CDTA, ○, ●; or 2 μmoles of Tris–ATP (pH 7.5) and 3 μmoles of $MgCl_2$, △, ▲; or 2 μmoles of Tris–ATP (pH 7.5), 8 μmoles of Tris–ADP (pH 7.5) and 11 μmoles of $MgCl_2$, □, ■; (in Panel B) 10 μmoles of CDTA and 0.02 μmole of KCl, △, ▲; or 10 μmoles of CDTA and 0.25 μmole of KCl, ×. Open symbols refer to the control, closed symbols refer to samples containing acetone. The reaction was stopped with acid at the times indicated and the amount of phospho-enzyme was estimated and corrected for blank phosphorylation as in Fig. 3. k is the rate constant for dephosphorylation in s^{-1}.

These experiments were repeated by using *N*-ethylmaleimide-treated enzyme which formed E_1–P only (Table V). Acetone inhibited the breakdown of E_1–P both in the absence ("spontaneous" breakdown) or in the presence of ADP. Acetone therefore stabilized both E_1–P and E_2–P against hydrolysis. The effect of aliphatic alcohols (from ethanol to *n*-heptanol), diethyl ether and *N*,*N*-dimethylformamide was similar.

Stabilization of the phospho-enzyme depended upon the concentration of the organic solvent as shown for *n*-butanol in Table VI. Also, as the concentration

of a solvent rose, so did the concentration of K^+ needed to reproduce the original dephosphorylation rate of E_2–P (not shown).

TABLE V

EFFECT OF ACETONE ON THE BREAKDOWN OF AN *N*-ETHYLMALEIMIDE-MODIFIED PHOSPHO-ENZYME

4 mg of plasma membrane protein were incubated in 4 ml of 10 mM imidazole–HCl (pH 7.45) containing 10 mM *N*-ethylmaleimide and 16 mM NaCl at 37 °C for 45 min. The reaction was stopped by adding β-mercaptoethanol to a final concentration of 50 mM. The membranes were washed three times by centrifugation and resuspension in imidazole–HCl buffer (pH 7.45). For phosphorylation, the reaction mixture contained at 0 °C in 0.8 ml: 16 μmoles of NaCl, 1 μmole of $MgCl_2$, 10 μmoles of imidazole–glycylglycine (pH 7.5), 0.19 mg of membrane protein and 1.32 mmoles of acetone when indicated. The reaction was started by the addition of 0.01 μmole of [^{32}P]ATP in 0.1 ml. After 5 s, further labeling was blocked with a combination of ATP, ADP, CDTA and KCl as indicated on the table. The reaction was stopped with acid, the amount of phospho-enzyme and the rate constants of dephosphorylation were estimated as in Fig. 4.

Radioactive phospho-rylation blocked with (mM)	*Rate constant of dephosphorylation (s^{-1})*	
	Control	*With 1.32 M acetone*
Tris–ATP (1)	0.092	0.009
Tris–ADP (4) + Tris–ATP (1)	0.30	0.09
CDTA (5)	0.097	0.009
CDTA (5) + KCl (0.3)	0.096	0.009

TABLE VI

RELATIONSHIP BETWEEN THE RATE OF HYDROLYSIS OF THE PHOSPHO-ENZYME AND THE CONCENTRATION OF *n*-BUTANOL

At 0 °C in 0.8 ml, the reaction mixture contained 32 μmoles of NaCl, 2 μmoles of $MgCl_2$, 10 μmoles of imidazole–glycylglycine (pH 7.5) and 0.23 mg of kidney membrane protein. The reaction started after the addition (in 0.1 ml) of 40 nmoles of [^{32}P]ATP. Further phosphorylation was blocked with 10 μmoles of Tris–CDTA (pH 7.5) or 10 μmoles of Tris–CDTA and 0.03 μmole of KCl. The amount of phospho-enzyme and the rate constant of hydrolysis were estimated as in Fig. 4.

Concn of n-butanol (mM)	*Concn of K^+ (mM)*	*Rate constant of hydrolysis (s^{-1})*
0	0	0.042
0	0.03	0.22
54	0.03	0.15
108	0.03	0.125
162	0.03	0.066
216	0.03	0.028

It is interesting to note that 3.3 M urea reduced the steady-state level of the phospho-enzyme (established at 40 μM ATP in 10 s) to 56% of the control but urea did not change the rate of dephosphorylation (not shown). 3.3 M urea reversibly inhibited 50% of the enzyme activity at 37 °C. Urea, therefore, inhibited the enzyme by a different mechanism than that of the organic solvents. This may reflect the difference in the way by which urea and organic solvents denature proteins. Other denaturing agents or procedures (*e.g.* heating) were not tested.

(*D*) *Rephosphorylation of the dephospho-enzyme*

In the previous experiments phosphorylation and dephosphorylation were studied separately. *In vivo* these two steps follow each other in the presence of all three ionic ligands of the enzyme: Na^+, K^+ and Mg^{2+}. (The reaction cycle was described earlier.) If both Na^+ and K^+ are present rephosphorylation of the dephospho-enzyme is partly limited by the rate of dissociation of the K^+–dephospho-enzyme complex[13]. Higher concentrations of ATP and Na^+ enhance this dissociation.

The effect of organic solvents on this step of the reaction was tested in the following way. First, the enzyme was phosphorylated with a low concentration of ATP (6 μM) and a relatively low concentration of Na^+ (8 mM). Phosphorylation reached steady state in 5 s. This level of the phospho-enzyme was taken arbitrarily as 100%. The level of the phospho-enzyme was reduced to about 40% with 0.01 mM Rb^+ (at 25 s). It was advantageous to use Rb^+ in place of K^+ because Rb^+ forms a more stable complex with the dephospho-enzyme than K^+. (The rate constant for the spontaneous dissociation of this complex is about 0.06 s^{-1} (ref. 13).) At 25 s 160 mM Na^+ and 1 mM Mg^{2+} were added to the control samples. This large excess

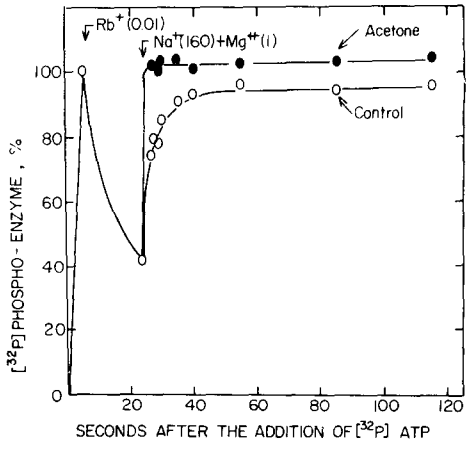

Fig. 5. Effect of acetone on the rephosphorylation of a dephospho-enzyme produced by Rb^+. At 0 °C in 0.4 ml the reaction medium contained 0.743 mg of membrane protein, 10 μmoles of imidazole–glycylglycine (pH 7.4) and 8 μmoles of NaCl. The reaction was started by adding 6 nmoles of [^{32}P]ATP and 0.5 μmole of $MgCl_2$ in 0.1 ml 5 s later. At 25 s, 160 μmoles of NaCl and 1 μmole of $MgCl_2$ were added in 0.4 ml to the control samples, (○); 160 μmoles of NaCl, 1 μmole of $MgCl_2$ and 0.2 ml of acetone (final concn 2.68 M) were added in 0.4 ml to the test samples (●). The reaction was stopped with acid at the times indicated. The amount of phospho-enzyme was estimated as described in Methods and Materials and the values were corrected for blank phosphorylation as in Fig. 3.

of Na^+ inhibited the recombination of Rb^+ with the dephospho-enzyme and initiated rephosphorylation. Rephosphorylation was almost complete but slow. It reached a steady-state level (about 94%) in about 15 s. If acetone was also added together with 160 mM Na^+ and 1 mM Mg^{2+}, rephosphorylation reached the steady-state level (about 104%) in about 1 s. (Fig. 5). Similar results were obtained with ethanol and *n*-butanol.

Inhibition of K^+-dependent p-nitrophenylphosphatase

K^+-*p*-nitrophenylphosphatase is a partial reaction of $(Na^+ + K^+)$-ATPase. Recently Albers and Koval[31] described inhibition of $(Na^+ + K^+)$-ATPase and concurrent activation of K^+-*p*-nitrophenylphosphatase by glycerol and dimethyl sulfoxide. These two compounds increased the steady-state level of the phospho-enzyme, decreased the rate of ADP–ATP exchange and the rate of splitting of acetyl phosphate. They did not change the sensitivity of the phospho-enzyme toward K^+ rather they increased the affinity for both Na^+ and K^+ and also the rate of splitting of *p*-nitrophenyl phosphate. In contrast, the solvents used in this work decreased the affinity for K^+. Furthermore, concentrations of acetone and *n*-butanol which inhibited $(Na^+ + K^+)$-ATPase by 50% also inhibited *p*-nitrophenyl phosphate activity, in the presence of K^+ alone by 66% and in the presence of Na^+, ATP and K^+ by 100%. (Table VII).

TABLE VII

INHIBITION OF K^+-NITROPHENYLPHOSPHATASE BY ACETONE AND *n*-BUTANOL

At 37 °C in 1 ml, the reaction mixture contained 0.043 mg of membrane protein, 3 μmoles of *p*-nitrophenyl phosphate, 3 μmoles of $MgCl_2$ and 10 μmoles of imidazole glycylglycine (pH 7.5). In addition, Medium A contained 10 μmoles of KCl; Medium B contained 2.5 μmoles of KCl, 10 μmoles of NaCl and 0.1 μmole of Tris–ATP (pH 7.5); and Medium C contained 0.25 μmole of ouabain. The estimation of K^+-*p*-nitrophenylphosphatase was described in Methods and Materials. $(Na^+ + K^+)$-ATPase activity of this membrane preparation was 3.4 units/mg protein.

Inhibitor	*Concn (M)*	*p-Nitrophenyl phosphate split (μmoles · mg protein · min^{-1})*		
		Medium		
		A	*B*	*C*
None	—	0.330	0.129	0.024
Acetone	1.32	0.125	0.019	0.023
n-Butanol	0.16	0.136	0.029	0.030

Possible relationship of the effect of organic solvents to the passive transport of K^+

In these experiments, the source of $(Na^+ + K^+)$-ATPase was a crude membrane preparation which formed vesicles as shown by electron microscopy[32]. It was, therefore, conceivable that K^+ had to diffuse across the walls of these vesicles in order to reach its site of action. If so, the organic solvents used in these experiments could have affected this movement of K^+. Inhibition of the passive movement of K^+ across the walls of vesicles could have accounted for the apparent decrease of affinity

for K^+. In order to test this possibility, an "ionophorous antibiotic"[33] was used. In one experiment 10 μg of valinomycin were added together with 1.32 M acetone or with 0.16 M *n*-butanol to 350 μg of membrane protein. $(Na^+ + K^+)$-ATPase was phosphorylated in the presence of 16 mM Na^+, 1 mM Mg^{2+} and 40 μM ATP and the rate of dephosphorylation was tested by adding 5 mM CDTA with 0.03 mM K^+ as in Fig. 4. Acetone or *n*-butanol alone slowed down the rate of dephosphorylation. 10 μg of valinomycin neither relieved nor aggravated this effect of acetone or *n*-butanol even though this amount of valinomycin is sufficient to induce a large diffusion of K^+ in red cells or in mitochondria[33]. It is therefore unlikely that acetone or *n*-butanol stabilized the phospho-enzyme by inhibiting passive K^+ movement (this experiment is not shown).

Relation between the hydrophobicity of organic solvents and their action on $(Na^+ + K^+)$-ATPase

The effectiveness of an organic solvent on $(Na^+ + K^+)$-ATPase strongly correlated with its hydrophobicity. Hydrophobicity was estimated from the octanol–water partition coefficient of the solvent. 1-Octanol was found to be the most suitable apolar liquid to serve as a model of the lipid phase of biological membranes[34,35]. In Fig. 6, the negative logarithms of the half-maximal inhibitory concentrations at 37 °C (pK_i) of 12 solvents are plotted against the logarithms of their octanol–water partition coefficients. The straight line in the graph is a least-squares regression line, calculated with the omission of the value for diethyl ether. The coefficient of correlation was +0.995, indicating a strong linear correlation between the pK_i values of these solvents and the logarithms of their partition coefficients. (pK_i for diethyl ether was omitted from the calculation of the regression line as no special precautions were taken to prevent evaporation of this extremely volatile solvent during the determination of the half-maximal inhibitory concentration. The pK_i value for diethyl ether is therefore not accurate.)

In the series of homologous saturated aliphatic alcohols hydrophobicity increases with the increasing carbon chain length. There was a positive linear correlation between the inhibitory constant, pK_i and the carbon chain length of these alcohols up to *n*-heptanol (Fig. 7). At even longer chain length, determination of pK_i became exceedingly difficult because of the low solubility in water of these alcohols. This may be the explanation for the deviation from linearity of the values for *n*-octanol and *n*-nonanol. Branched isomers (*e.g.* isopropanol and isobutanol) were less effective inhibitors than the corresponding normal alcohols (dark circles in Fig. 7). It was assumed that primarily the straight portion of the alcohol chain would determine the hydrophobic properties of the molecule[36]. More recently it was pointed out that permeability of the plasma membrane was higher for straight chain isomers of aliphatic amides because the branched chain hindered the diffusion of these molecules within the membrane[37]. Since $(Na^+ + K^+)$-ATPase is embedded in the plasma membrane both of these mechanisms might have weakened the inhibition by branched chain alcohols.

By assuming: (a) equilibrium between the native and inhibited enzyme, and (b) stoichiometric combination of the solvent molecules with $(Na^+ + K^+)$-ATPase, the free energy of formation of the inhibited form can be calculated from the inhibitory constant (K_i). The assumption of equilibrium is supported by the reversibility of this

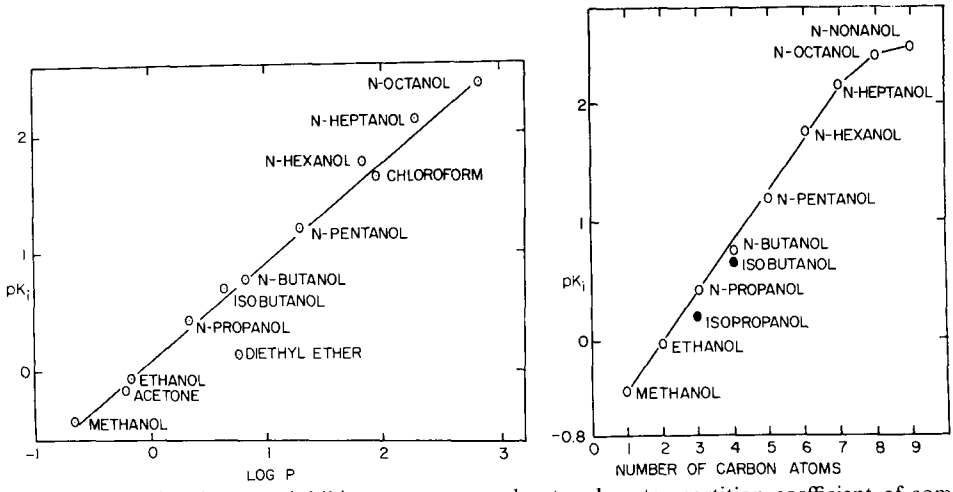

Fig. 6. Correlation between inhibitory potency and octanol–water partition coefficient of some organic solvents. The logarithms of the octanol–water partition coefficients (log P) of the solvents were taken from ref. 44. The negative logarithms of half-maximal inhibitory concentrations (pK_i) of these solvents were estimated as in Table I. The slope of the straight line in this graph was calculated by the least-squares method but with the omission of the value for diethyl ether.

Fig. 7. Correlation between inhibitory potency and carbon chain length of aliphatic alcohols. The straight line in this graph connects pK_i values of normal alcohols plotted against the number of carbon atoms in the chain (open symbols). pK_i values of branched isomers were placed on the graph according to the number of carbon atoms in the molecule (closed symbols). pK_i was estimated as in Fig. 6.

inhibition at low solvent concentrations. (No specific molecular mechanism of inhibition has to be considered for this calculation.) From the difference of the free energy values or from the slope of the straight line in Fig. 7 (drawn across the values for normal alcohols) the change in free energy due to addition of one CH_2 group can be estimated. For $(Na^+ + K^+)$-ATPase this value was -600 cal per mole per CH_2 group. As a comparison, for other enzymes the free energy difference per CH_2 group of the inhibiting alcohol was (in cal/mole): -650 for lipoxigenase[38], -560 for pepsin[39], -820 for pancreatic lipase[40] (where the alcohols were absorbed on the substrate). Complete transfer of an alcohol from aqueous into hydrophobic environment results in a free energy change of about -830 cal/mole per CH_2 group. One might assume, therefore, that this transfer was not complete in the inhibition of $(Na^+ + K^+)$-ATPase by alcohols, *i.e.* that the assumed binding site was not completely hydrophobic.

From K_i for *n*-pentanol and from its temperature dependence (Fig. 8) the thermodynamic parameters of formation of the inhibited enzyme can be estimated. Two assumptions were made for this calculation. First: the native and the inhibited enzyme were in equilibrium; second: the inhibited enzyme was a single molecular species combined stoichiometrically with *n*-pentanol. The first assumption is supported by the reversibility of the inhibition, the second assumption could not be tested directly. On these premises, the following values were obtained: free energy of formation (ΔG_f°) was -1850 cal/mole, free enthalpy of formation (ΔH_f°) was 4880 cal/mole and the free entropy (ΔS_f°) was 22.6 e.u.; *i.e.* the transfer of *n*-pentanol

from the aqueous medium to the postulated binding site(s) on the enzyme was an endothermic process and resulted in a large increase in entropy. Similar thermodynamic changes characterize the transfer of a non-polar compound from water into a non-polar solvent[41], and therefore this finding is consistent with a primarily hydrophobic interaction between the enzyme and *n*-pentanol. Theoretically it has also been predicted that ΔH_f° and ΔS_f° should decrease and eventually become negative at higher temperatures[41], as it was demonstrated for lipoxygenase[38]. Since $(Na^+ + K^+)$ ATPase was inactivated too rapidly above 45 °C, this test could not be carried out.

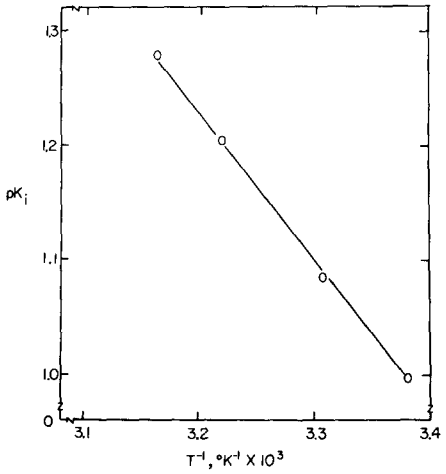

Fig. 8. Temperature dependence of inhibition of $(Na^+ + K^+)$-ATPase by *n*-pentanol, van't Hoff plot. The apparent half-maximal inhibitory concentration of *n*-pentanol (K_i) at various temperatures was estimated as described in the legend of Table I. The activity of the enzyme was estimated by Method A.

DISCUSSION

The organic solvents used in these experiments interfered with several steps of the reaction cycle of $(Na^+ + K^+)$-ATPase. Their actions can be summarized as effects on the binding of different ligands to the enzyme and stabilization of the phospho-enzyme.

The solvents increased the affinity for Na^+ and ATP and decreased the affinity for K^+ as estimated from kinetic parameters of the hydrolysis of ATP (Table II). The solvents reversed inhibition by K^+ at low concentrations of ATP (Fig. 2) and also relieved inhibition by K^+ of the binding of ATP to the enzyme (shown by pulse labeling experiments in Fig. 3). All these phenomena could be explained by assuming that the organic solvents facilitated the dissociation of K^+ from the enzyme. Direct testing of specific K^+ binding to the crude enzyme preparation did not seem feasible but the measurement of the rate of rephosphorylation provided an indirect estimate of the dissociation of K^+ (Fig. 5). At low concentrations of ATP (6 μM in this experiment) Rb^+ forms a relatively stable complex with the dephospho-enzyme after splitting the phospho-enzyme. The dissociation of this Rb^+–dephospho-enzyme complex limits the rate of rephosphorylation, but after dissociation, the free Rb^+

has probably little or no effect on rephosphorylation because of the high Na^+/Rb^+ ratio (16000/1). (This is shown in Fig. 9 of ref. 13.) Acetone markedly accelerated this rephosphorylation. By assuming a simplified reaction scheme for $(Na^+ + K^+)$-ATPase (Eqn 1) the rate constant for rephosphorylation can be estimated.

$$E + \text{ATP} \xrightarrow{k_1} E\text{–}P \xrightarrow{k_2} E + P_i \quad (1)$$

In this scheme k_1 is the rate constant for phosphorylation, k_2 is the rate constant for dephosphorylation. (Conversion of E_1–P to E_2–P and the addition of different ligands to the enzyme are not considered.) The rate constant for approaching the steady-state level is $(k_1 + k_2)$, fractional steady-state level (F_s) is $k_1/(k_1 + k_2)$. In the control experiment the first order rate constant of approaching steady-state level of E–P, *i.e.* $(k_1 + k_2)$, was 0.17 s^{-1}, F_s was 0.89 and thus k_1 was 0.15 s^{-1}. In the presence of acetone k_2 became negligible (less than 0.01 s^{-1}) and k_1 was estimated to be larger than 1 s^{-1} (More exact estimation of k_1 was not possible with a manual procedure.) Estimated directly with a rapid mixing apparatus, k_1 was 2.1 s^{-1} in the experiments of Kanazawa *et al.*[26]. Therefore, acetone appears to have restored the uninhibited rate of phosphorylation, probably by dissociating Rb^+. (As the rate of phosphorylation could not be measured directly, it is not known whether acetone could increase the rate of phosphorylation in the presence of Mg^{2+} and Na^+ alone.)

Organic solvents stabilized the phospho-enzyme both in the presence or in the absence of K^+ (Fig. 4 and Table IV). It is particularly important that the solvent stabilized also E_1–P (formed by an *N*-ethylmaleimide-treated enzyme) which was completely insensitive to K^+ (Table V). The organic solvents, therefore, directly affected the hydrolytic step, not only indirectly by antagonizing K^+.

Since the partition coefficients and the half-maximal inhibitory concentrations of the solvents correlated with each other (Fig. 6), inhibition by these solvents implies partition between the aqueous medium and a hydrophobic region on the enzyme. This region may comprise hydrophobic side chains of amino acids and some of the membrane lipids which are essential for the activation of the enzyme[32]. Transfer of a solvent from water into this hydrophobic region could change the conformation of the enzyme and/or the state of some of the water bound to the enzyme. But, different organic solvents inhibited both the effects of K^+ on the enzyme (Figs 2, 3, 4, 5) and the hydrolysis of the phospho-enzyme even in the absence of K^+. This concurrent inhibition of these two processes may indicate that they have a common mechanism which can be affected by organic solvents. The following hypothesis is but one possible visualization of this mechanism.

In this model the phosphate acceptor site of $(Na^+ + K^+)$-ATPase would be near to a hydrophobic region which limits the access of water. Spontaneous hydrolysis of the phospho-enzyme would be slow for this reason. K^+ at its site would change the conformation of the enzyme and would increase the access of water to the phosphate acceptor site. On the other hand combination of organic solvents with the hydrophobic region would displace water from the phosphate acceptor site. By assuming that K^+ and water mutually assist each other in their binding to the corresponding sites, the expulsion of water from the phosphate acceptor site would enhance the dissociation of K^+ from its site. Dissociation of K^+, in turn, facilitates the binding of ATP and Na^+ to the enzyme. Thus, the binding of K^+ would affect and would be affected by the state of water at the phosphate acceptor site. This model

accounts for the effects of apolar solvents on both dephospho- and phospho-enzyme without postulating the formation of different molecular species of the enzyme in the presence of solvents. This idea is also supported by the experiment of Ahmed *et al.*[42]. From a kinetic analysis of the action of deuterated water on $(Na^+ + K^+)$-ATPase and K^+-*p*-nitrophenylphosphatase activity they concluded that "...in the presence of ^2H_2O the disturbance in the hydrophobic bonding in the region of E_2–P results in a change in its conformation... in such a manner that the affinity of K^+ to react with this phosphorylated form is increased."

In the experiments reported here, the hydrophobicity of 15 organic solvents correlated with their effectiveness. In two extensive studies, the narcotic[43] and hemolytic[44] efficiency of more than 100 organic compounds correlated with their hydrophobicity. But does hydrophobicity alone determine effects of a solvent on $(Na^+ + K^+)$-ATPase? No. In the experiments of Albers and Koval[31] two organic solvents, glycerol and dimethyl sulfoxide, affected $(Na^+ + K^+)$-ATPase and K^+-*p*-nitrophenylphosphatase quite differently than did the solvents reported in this paper (see also Results and Table VII). The exact cause of this difference is not known. Glycerol and dimethyl sulfoxide, unlike most organic solvents, may not reduce but rather promote the hydration of proteins as demonstrated by studying the protection of the cellular structure and of macromolecules by these agents against freeze–thaw injury[45].

There was no relationship between the inhibition of $(Na^+ + K^+)$-ATPase and the dielectric strength of the incubation medium. The concentration of the more powerful inhibitors (long chain alcohols, chloroform) was too low to change the dielectric strength of the medium significantly. Also, if inhibition were due to dielectric changes, isobutanol should be a more potent inhibitor than *n*-butanol; opposite to what was found (Fig. 7). (In contrast trypsin and chymotrypsin activity depend on the dielectric strength of the medium[46].)

Grisham and Barnett[47] observed that high concentrations of ethanol and *tert*-butanol inactivated $(Na^+ + K^+)$-ATPase irreversibly by disrupting primarily the phospholipid bilayer close to the enzyme but they had little or no effect on the enzyme protein itself. It is not known whether similar changes take place during reversible inhibition of the enzyme by organic solvents which was reported in this paper.

ACKNOWLEDGEMENTS

This work was supported by Grant 5 RO1 HL 01974 from the National Heart and Lung Institute, National Institutes of Health, United States Public Health Service. I am very grateful to Dr Robert L. Post for his suggestions and for his criticism of the manuscript, to Mr James Brineaux for preparing the membranes and to Mrs Jean Sorrells for performing some of the experiments.

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