Journal of Supramolecular Structure 3:479–497 (1975)

SYNTHESIS OF ADENOSINE TRIPHOSPHATE BY WAY OF POTASSIUM-SENSITIVE PHOSPHOENZYME OF SODIUM, POTASSIUM ADENOSINE TRIPHOSPHATASE

Robert L. Post, Gotaro Toda, Shoji Kume, and Kazuya Taniguchi

Department of Physiology, Vanderbilt University Medical School, Nashville, Tennessee

The sodium and potassium ion pump is an intrinsic enzyme of plasma membranes. In these experiments it was driven backward in a transient two-step operation involving, first, phosphorylation of the enzyme from inorganic phosphate, and second, transfer of the phosphate group from the enzyme to ADP upon addition of a high concentration of Na^+. There was no evidence of a significant concentration gradient across the membranes. Na^+ presumably reached the solutions on both faces of the membrane simultaneously and provided the energy for synthesis simply as a consequence of ligand binding. An interaction free energy between the free energy of the binding of Na^+ and the free energy of hydrolysis of the phosphate group on the enzyme was estimated. The experiments also suggested a feature of the transport mechanism. This is control by phosphorylation of access pathways from the solutions in contact with the faces of the membrane to an active center for cation binding. In the dephosphoenzyme access would be to the intracellular solution and in the phosphoenzyme access would be to the extracellular solution.

INTRODUCTION

ATP has been synthesized by driving the Na-K pump backward in a steady state in intact cells with energy from concentration gradients of Na^+ and K^+ across the membrane (1). In the experiments reported here, ATP was synthesized transiently by the pump in leaky membranes probably without a gradient. In a two-step, one-shot operation, a phosphoenzyme was first prepared from inorganic phosphate (P_i) and subsequently was treated with ADP and a high concentration of Na^+ to release ATP. If Na^+ acted in the absence of a gradient, then binding of Na^+ was sufficient. Translocation involves binding

This paper was presented at a study week on "Biological and Artificial Membranes and Desalination of Water," held by the Pontifical Academy of Sciences, Vatican City, April 12–19, 1975.

G. Toda and S. Kume are now at First Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan.
K. Taniguchi is now at Department of Pharmacology, School of Dentistry, Hokkaido University, Sapporo, Japan.

 © 1975 Alan R. Liss, Inc., 150 Fifth Avenue, New York, N. Y. 10011

of substrate from a solution in contact with one face of a membrane and release of the same molecule as product into a solution in contact with the other face of the membrane. If binding of Na^+ was sufficient, then the translocation step was partially fractionated. These experiments also help to clarify the reaction sequence of phosphorylation and dephosphorylation, since the phosphoenzyme prepared from P_i had the same characteristics as phosphoenzyme prepared from ATP.

Per cycle, the sodium and potassium ion pump of plasma membranes transports approximately three Na^+ outward and two K^+ inward and hydrolyses the terminal phosphate bond of one molecule of intracellular MgATP (Fig. 1). Evidence comes from erythrocytes, nerve, and muscle (1). Na^+ is unique as a substrate for net outward transport. To drive the pump backwards, Garrahan and Glynn (2) and others (1) prepared erythrocytes containing only K^+ intracellularly in a medium containing only Na^+. Na^+ was transported inward, K^+ outward, and ATP was synthesized at a rate about 1.4% the capacity of the pump to run forward under optimal conditions.

In preparations of broken or leaky membranes, the Na^+-, K^+-dependent ATPase activity of the pump persists and is convenient for study even though intracellular and extracellular phases are no longer separate. This enzyme is abbreviated Na, K-ATPase (EC 3.6.1.3). It is an intrinsic enzyme of plasma membranes (3).

Another reason for running the pump backward in the broken membrane preparation concerns the reaction sequence of phosphoenzyme. Na^+, K^+-ATPase accepts a phosphate group from ATP in the presence of Na^+ (uniquely) and Mg^{2+}. The resulting phosphoenzyme is insensitive to ADP and exchanges its phosphate group with inorganic phosphate, P_i (the rate constant is about 0.1 sec^{-1} at 0°C). We have called such a reactive state of the phosphoenzyme E_2-P (4). In contrast, after partial poisoning with N-ethylmaleimide or oligomycin, the enzyme shows Na^+-dependent ATP $\rightleftharpoons$ ADP exchange and the phosphoenzyme is easily split by ADP (3). We have called such a reactive state of phosphoenzyme E_1-P. We have supposed in the native enzyme that a similar reactive state is a precursor to E_2-P in accordance with the initial suggestion of Albers and co-workers. However, the evidence is not definitive and a precursor-product relationship between E_1-P and E_2-P is controversial (5). In order to seek evidence for interconversion of these reactive forms in the native enzyme, we attempted reversal of the reaction from E_2-P (prepared directly from P_i) to E_1-P as shown by synthesis of ATP.

From various partial reactions of the Na, K-ATPase, a reaction sequence for phosphorylation and dephosphorylation of the enzyme can be assembled. It reflects in part the work and conclusions of several laboratories but is still controversial (5). It illustrates our working hypothesis (Fig. 2).

In this scheme "E" represents the enzyme. The most important feature of the scheme is the distinction between two reactive forms of the enzyme, E_1 and E_2. E_1 is phosphorylated reversibly from ATP in the presence of Na^+ to give E_1-P. In contrast, E_2 is phosphorylated reversibly from inorganic phosphate, P_i in the absence of Na^+ to give a different reactive form of the phosphoenzyme E_2-P. Actually, E_2-P is subdivided further into reactive states of which two, sensitive E_2-P and K · E_2-P, are shown here. Sensitive E_2-P is the predominant product of phosphorylation from ATP at low concentrations of Na^+ (about 10–100 mM). It is called "sensitive" because it is extremely sensitive to K^+ and rapidly combines with it to become K^+-complexed E_2P. Sensitive E_2-P exchanges its

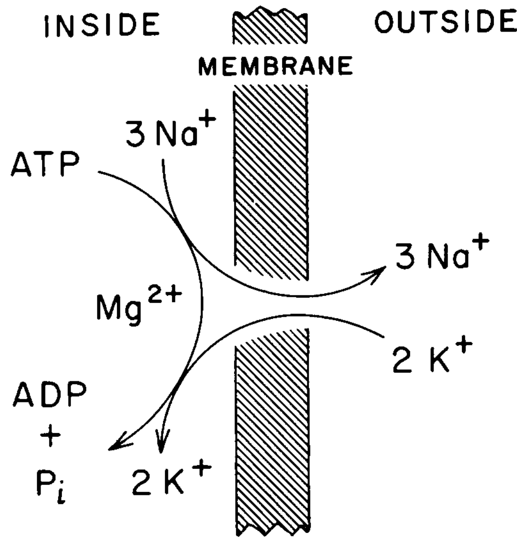

Fig. 1. Stoichiometry and sidedness of the sodium and potassium ion pump in the plasma membranes of animal cells.

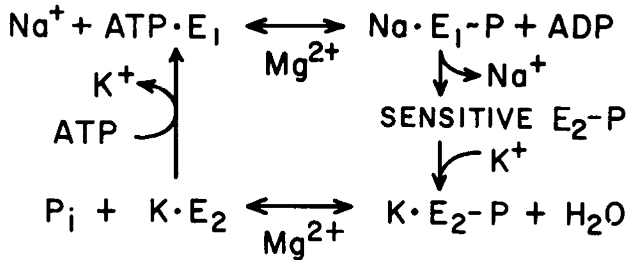

Fig. 2. Hypothetical reaction sequence for phosphorylation and dephosphorylation of Na, K-ATPase. "E" represents the enzyme and the subscripts 1 and 2 indicate major reactive forms. "Sensitive" refers specifically to sensitivity to K^+ or its congeners. Reproduced from reference 15.

phosphate group with P_i with a rate constant of about $k = 0.06\ (sec)^{-1}$, but $K \cdot E_2$-P exchanges much more rapidly with $k > 1\ (sec)^{-1}$ (4).

The strategy for synthesis of ATP was first the preparation of sensitive E_2-P from $^{32}P_i$. This was done by Dr. Toda in this laboratory. The second step was the addition of ADP and a high concentration of Na^+ to sensitive E_2-P with recovery of $[^{32}P]$ATP in the supernatant after the reaction was stopped with acid. This was done by Dr. Taniguchi (6). It was important to avoid K^+ or its congeners which prevented synthesis presumably by forming $K \cdot E_2$-P rapidly at low concentrations.

METHODS

The Na, K-ATPase was prepared in a crude suspension of membranes obtained from a homogenate of guinea pig kidney by differential centrifugation. As part of the purification procedure, the membranes were kept overnight at 4°C in 1.3 M urea. As the second part of the procedure, the membranes were treated with 2 M NaI at 0°C for 45 min. These treatments greatly reduced ATPase activity insensitive to Na^+ and K^+ and also adenylate kinase activity, which are contaminants in this preparation. These treatments are sufficiently vigorous that one would expect to obtain leaky vesicles. Three tests showed no evidence of significant impermeability to Na^+ (6).

For phosphorylation, 1–3 mg of membrane protein were incubated at 0°C and pH 7.5 with 10 or 20 μmoles of imidazole glyclyglycine in a final volume of 1 ml in the presence of Na^+, K^+, or neither. Usually, addition of [γ-^{32}P] ATP or $^{32}P_i$ was followed by addition of Mg^{2+} 3–20 sec later to start the reaction. Additions were in volumes of 0.1 ml to produce the final concentrations indicated. After an interval of 3 sec to 1 min (as indicated in the figures), the reaction was stopped with acid. After centrifugation and separation of the supernatant the denatured membranes were usually digested with pepsin. This procedure released radioactive phosphopeptides overlapping the active site into the supernatant for subsequent isolation by paper electrophoresis. The amount of phosphoenzyme estimated in this way is usually expressed as a percentage of maximal phosphorylation as obtained from [^{32}P]ATP under standard conditions. In this system final purification takes place after denaturation of the enzyme, rather than before. Specific details and alternative procedures are described elsewhere (4, 6). In experiments where ATP was synthesized from $^{32}P_i$ and ADP, the first supernatant was subjected to column chromatography to isolate [^{32}P]ATP (6, 7). In experiments where [^{32}P]dATP was synthesized, it was isolated from the supernatant by column chromatography on polyethylene imine-Avicel in the presence of borate followed by thin layer chromatography on polyethylene imine-cellulose. Final contamination of dATP with ATP was less than 0.02%.

RESULTS

K^+-Sensitive Phosphoenzyme

After Na^+, K^+-ATPase was first phosphorylated from ATP, the rate of dephosphorylation could then be estimated by interrupting further phosphorylation and observing the subsequent rate of disappearance of the radioactive phosphoenzyme. Phosphorylation from [^{32}P]ATP was interrupted either by a chase of cold ATP or by chelation of Mg^{2+} with an excess of a chelator such as EDTA or CDTA (cyclohexylenediaminetetraacetic acid). In order to observe the sensitivity to K^+, KCl was added to the phosphoenzyme in the presence of excess EDTA. Dephosphorylation was immediate and complete in less than 4 sec. In the absence of K^+ the apparent monomolecular rate constant at 0°C was $k = 0.06$ sec^{-1}. The high specificity of the reaction is shown by the ratio of [K^+] to [Na^+] which was 1 : 160 (Fig. 3).

Sensitivity of this reactive state of the enzyme to ADP was tested by addition of deoxy-ADP during a chase with unlabeled ATP. There was no effect (Fig. 4). Deoxy-ADP was substituted for ADP since it is a poor substrate for adenylate kinase, which may con-

taminate the preparation. This experiment serves as a control for the next two experiments.

Is E_1-P a Precursor to E_2-P?

This question is controversial (5). In order to make an experimental test, the enzyme was incubated with [^{32}P]ATP and Na^+. This allowed ATP to bind to its active site. The reacton was started with Mg^{2+} and a chase of unlabeled ATP. A pulse of radioactive phosphoenzyme was obtained from the bound ATP (Fig. 5). The chase of unlabeled ATP prevented further phosphorylation from [^{32}P]ATP after the initial burst. In order to test for the presence of a transient state of the phosphoenzyme which might be sensitive to ADP, dADP was added with the chase of unlabeled ATP. This addition reduced the initial amount of [^{32}P]phosphoenzyme by 270 pmoles out of a total of 1,320 pmoles and did not affect the rate of dephosphorylation (Fig. 5). About 20% of the initial [^{32}P]phosphoenzyme in this experiment was transiently in a state in which it was sensitive to dADP. Deoxy-ADP cannot be more effective than unlabeled ATP in preventing phosphorylation from [^{32}P]ATP.

In order to find out if the deficiency in phosphoenzyme was due to a reversal of phosphorylation, the supernatant fluids were analyzed for [^{32}P]dATP. There were about 270 pmoles more dATP (Fig. 6) when the reaction was started with Mg^{2+} and a chase of unlabeled ATP (Fig. 5) then when the same chase was added to the preformed phosphoenzyme (Fig. 4). Since 20% of the enzyme which accepted a phosphate group from ATP returned it to dADP immediately after phosphorylation was initiated with Mg^{2+} (Fig. 5) and did not do so 5 sec later (Fig. 4), it passed from a state of sensitivity to ADP, E_1-P, to an ADP-insensitive state, E_2-P.

But why only 20%? According to a long-standing hypothesis (3), conversion of E_1-P to E_2-P requires a second addition of Mg^{2+} to the enzyme. Perhaps adjustment of the concentration of Mg^{2+} would improve the evidence. However, Klodos and Skou observed no effect of changes in the concentration of Mg^{2+} during dephosphorylation following addition of excess ADP (8). Furthermore, they found an error in the interpretation of experiments from this laboratory (which they confirmed). These experiments were previously interpreted to support the hypothesis (9). Consequently, free Mg^{2+} does not appear to be required for conversion of E_1-P to E_2-P (8). Rather than addition of Mg^{2+}, release of Na^+ from the enzyme appears to be the significant step, as will be shown.

Addition of dADP to the phosphoenzyme (along with a chase of unlabeled ATP) produced threefold more dATP in the presence of 200 mM Na^+ than in the presence of 8 mM Na^+ (Fig. 7). These experiments were done by Dr. Kume. To explain the incompleteness of the action of dADP, one might suppose that Na^+ was released about as rapidly as ADP from Na · E_1-P · ADP and that this release prevented reversal of the reaction. One might suppose further that the affinity of the phosphoenzyme for Na^+ was much less than that of the dephosphoenzyme.

Rather than pursue the forward reaction, we began to investigate the reverse reaction after we found that the native enzyme spontaneously accepts a phosphate group from P_i (4, 10).

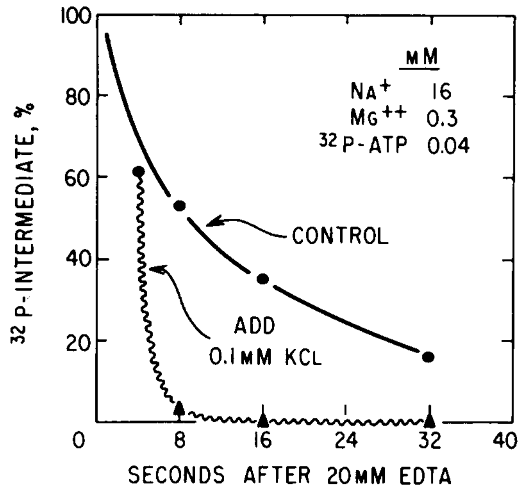

Fig. 3. Sensitivity to K^+ of phosphoenzyme prepared from ATP. "^{32}P-intermediate" refers to the phosphoenzyme. The initial conditions at 0°C and pH 7.6 are shown in the inset. Reproduced from reference 16.

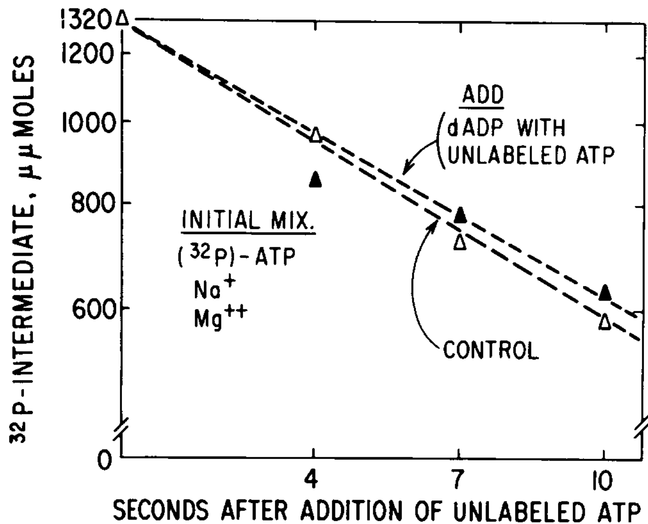

Fig. 4. Insensitivity to dADP of phosphoenzyme prepared from ATP. In a final volume of 2 ml the reaction mixture contained membranes, partly purified only by treatment with urea but not NaI, 20 μmoles of imidazole glyclyglycine, 4 μmoles of $(Tris)_3CDTA$, 6 μmoles of $MgCl_2$, and 16 μmoles of NaCl. The reaction was started with 0.04 μmoles of [^{32}P]ATP. Five sec later a chase of 2 μmoles of unlabeled Mg · ATP was added without (△) or with (▲) 4 μmoles of Mg · dADP. "^{32}P-intermediate" refers to the phosphoenzyme.

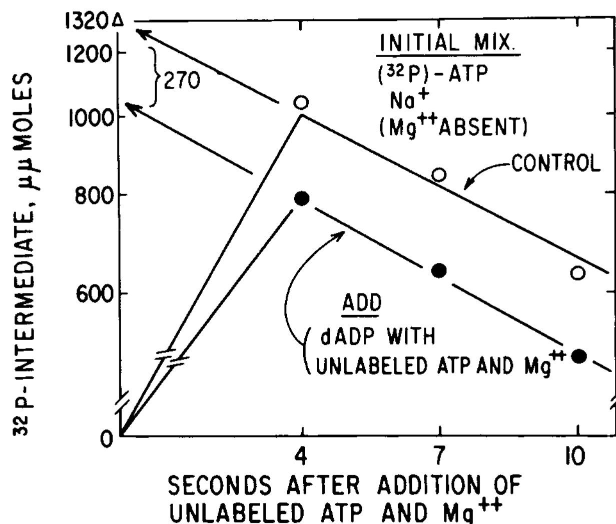

Fig. 5. Partial inhibition by dADP of a transient burst of phosphorylation from [^{32}P]ATP bound beforehand at its active site. The reaction was conducted as in Fig. 4 except that 6 μmoles of $MgCl_2$ were omitted from the initial mixture and were added with the chase of unlabeled Mg · ATP without (○) or with (●) Mg · dADP.

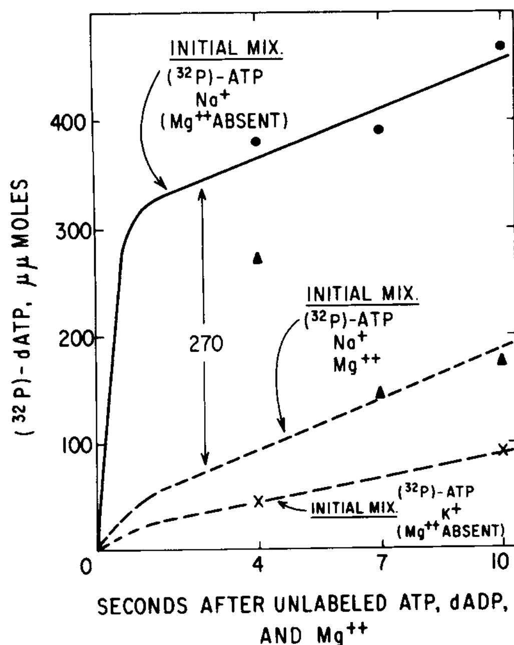

Fig. 6. Transient synthesis of dATP from [^{32}P] phosphoenzyme preliminary to appearance of K^+-sensitive phosphoenzyme. The closed circles (●) show data from the experiment of Fig. 5. The closed triangles (▲), show data from the companion experiment of Fig. 4. The crosses (X) show data from control samples in which Na^+ was replaced with equivalent K^+.

Formation of K^+-Sensitive Phosphoenzyme from P_i

In order to investigate the effects of Na^+ and K^+ on phosphorylation from P_i, the enzyme was incubated with P_i and the reaction was started with Mg^{2+} alone or with Mg^{2+} and Na^+ or K^+. With Mg^{2+} alone, phosphorylation was slow; addition of Na^+ inhibited phosphorylation, and addition of K^+ stimulated it (Fig. 8). It turned out that the phosphoenzyme formed in the presence of K^+exchanged its phosphate group with P_i much more rapidly than did that formed in the absence of K^+ (see Fig. 9). This rapid exchange was taken as an identifying characteristic of K^+-complexed phosphoenzyme of $K \cdot E_2$-P (4). However, phosphoenzyme from P_i with Mg^{2+} alone was insensitive to K^+ (Fig. 9) and therefore was not suitable for demonstrating reversal of the normal reaction. We called this reactive state "insensitive phosphoenzyme" since it was also insensitive to ADP (4).

In Fig. 9 it is significant that formation of insensitive phosphoenzyme was more extensive than in Fig. 8 and was also diphasic. The significant difference in the procedure is that in Fig. 9 the membranes were washed once in 1 mM $MgCl_2$ at 0°C and neutral pH by centrifugation and resuspension. After one wash the $MgCl_2$ was removed and the experiment was performed. In the experiment of Fig. 8 the membranes were not washed. Since this washing increased the yield of phosphoenzyme in the absence of K^+ or Na^+, we continued the procedure in subsequent experiments. Washing probably partially transforms E_1 into E_2.

Sensitive phosphoenzyme from ATP is slowly converted into insensitive phosphoenzyme due to an attack by Mg^{2+} (4). Perhaps the insensitive phosphoenzyme in Fig. 9 really began as sensitive phosphoenzyme and then was attacked by the Mg^{2+}. Experiments at short times were indecisive. In order to try a different ligand, washed membranes were incubated with P_i and the reaction was started with Fe^{2+}. After 3 sec three-fourths of the resulting phosphoenzyme was sensitive to K^+ (Fig. 10). It appeared that formation of sensitive phosphoenzyme was taking place and that the problem was to protect it from a secondary effect of Mg^{2+}.

Na^+ protects sensitive phosphoenzyme (made from ATP) from attack by Mg^{2+} (4) but it also inhibits phosphorylation from P_i. In order to estimate the rate of development of the inhibitory effect of Na^+ in the washed membranes, Na^+ was added at various times. The fast and slow components of phosphorylation (Fig. 9) responded to inhibition by Na^+ at different rates. The slow component was inhibited immediately (as in Fig. 8), but the fast component was inhibited relatively slowly. Inhibition required more than 5 sec and less than 10 min (Fig. 11). Six experiments showed that the apparent monomolecular rate constant for inhibition by NaCl was between 0.05 and 0.16 $(sec)^{-1}$. It was not very consistent. The relative slowness of the inhibitory action of Na^+ on washed enzyme permitted its use to protect sensitive phosphoenzyme from Mg^{2+}.

In order to estimate the stability of sensitive phosphoenzyme under these conditions, phosphoenzyme was treated with CDTA and K^+ and the fraction sensitive to K^+ was estimated at 4, 12, and 48 sec. Sensitive phosphoenzyme was practically intact at 4 sec (Fig. 12).

In order to compare sensitive phosphoenzyme prepared in this way from P_i with sensitive phosphoenzyme prepared from ATP, the two phosphorylation procedures were applied to the same enzyme preparation. The rate of dephosphorylation of sensitive phosphoenzyme in the presence of various concentrations of K^+ or its congeners was

estimated. Sensitive phosphoenzyme from both sources, [^{32}P]ATP or $^{32}P_i$, was identical. This preparation of sensitive phosphoenzyme from P_i was therefore suitable as a starting material for attempting reversal of the reaction.

Synthesis of ATP

In order to synthesize ATP, sensitive phosphoenzyme was prepared as in Fig. 12. Four sec later ADP and excess CDTA were added without or with supplemental Na^+. The purpose of the CDTA was to trap all the Mg^{2+} which might be formed and to protect the ATP from enzymatic attack. It also inhibited adenylate kinase. At 16 mM Na^+ the phosphoenzyme disappeared in almost the usual manner (Figs. 3 and 4) and no ATP appeared. At 176 mM Na^+ the rate of disappearance of the phosphoenzyme was more rapid and a small amount of ATP appeared slowly and then remained constant. The yield of ATP was about 20% of the initial amount of phosphoenzyme. The rest of the phosphoenzyme hydrolyzed spontaneously in a competing reaction. Later experiments showed that CDTA was not necessary in this reaction system. It did stabilize the ATP but reduced the yield by about 25% (6). Stabilization was more important than maximal yield so that CDTA was used in later experiments.

Clearly, a high concentration of Na^+ was important for synthesis. In further experiments the concentration of Na^+ was varied (Fig. 13) and the apparent monomolecular rate constant for transfer of the phosphate group from the phosphoenzyme to ADP (at a saturating concentration) was estimated (Fig. 14). The half-maximal concentration of Na^+ was about 0.6 M. The rate of rise of the rate relative to the Na^+ concentration was steeper than in a simple Michaelis-Menten relationship.

With respect to the experiments with dADP, particularly that in Fig. 7, the rate of synthesis of dATP was slower than that of ATP according to Fig. 14. In Fig. 14 at 16 mM Na^+ the rate constant was about 1%/sec and at 200 mM Na^+ it was about 15%/sec. In Fig. 7 the appearance of dATP was biphasic. The rapid component was considered to come directly from E_1-P. Part of the slow component could have come from conversion of E_2-P to E_1-P. However, at 8 mM Na^+ no Na^+-dependent slow component was detected (<0.2%/sec) and at 200 mM Na^+ the Na^+-dependent slow component was only about 1%/sec. It is not immediately apparent which features of the system in Fig. 7 made it so much slower.

Control Experiments

ATP was not synthesized from K^+-complexed phosphoenzyme nor in the presence of the specific cardioactive steroid inhibitor, ouabain (6, 7). ATP was not synthesized when the order of addition of ligands was changed. In a reaction system with 160 mM Na^+ and 1 mM ADP in which 22% of the initial amount of phosphoenzyme was converted to ATP, neither phosphoenzyme nor ATP appeared when the Na^+ or the ADP was added to the enzyme about 10 min before P_i was added. When K^+ or its congeners, Li^+, NH_4^+, Rb^+, Cs^+, or Tl^+, were added to phosphoenzyme together with 160 mM Na^+ at concentrations which were estimated (from other experiments) to produce equivalent acceleration of dephosphorylation of K^+-sensitive phosphoenzyme, then approximately equivalent partial inhibition of ATP synthesis was obtained in each case (7). Consequently, ATP synthesis appears to depend on competition between Na^+ and K^+ for occupancy of sensitive

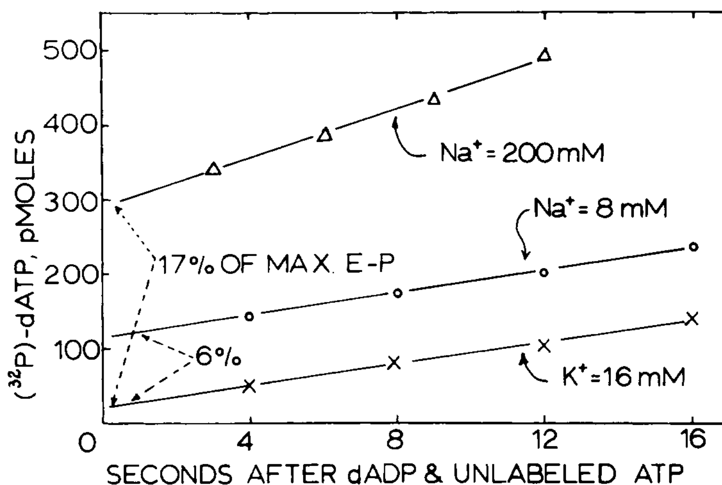

Fig. 7. Effect of Na^+ concentration on synthesis of dATP from the same amount of phosphoenzyme prepared from [^{32}P]ATP in a steady state. The slope of the line in K^+ shows the rate of dADP ⇌ ATP exchange after the specific activity of the [^{32}P]ATP was reduced 50-fold by the chase. The reaction system was similar to that in Fig. 4.

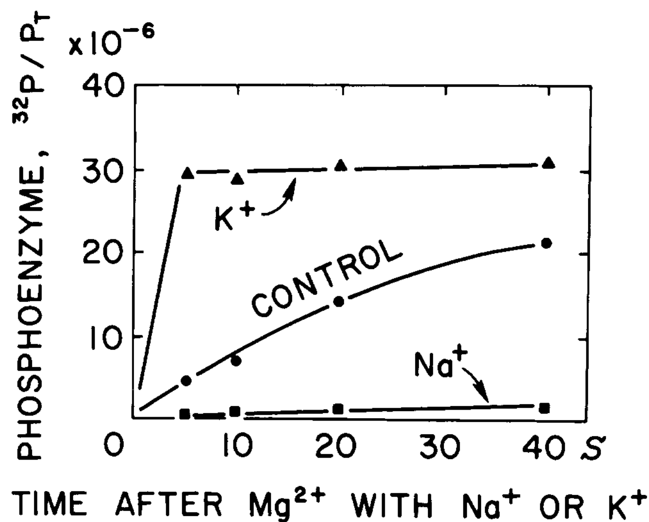

Fig. 8. Phosphorylation from P_i of unwashed membranes. The membranes were incubated with 1 mM $^{32}P_i$ for 10 sec before zero time. [Mg^{2+}] = 2 mM, [Na^+] = [K^+] = 16 mM. In the control Na^+ and K^+ were omitted. "P_T" refers to total phosphorus as an estimate of the amount of membranes in the mixture. Maximal phosphorylation was not estimated but was about 100–200 pmoles ^{32}P/μmole P_T as judged from other experiments. The level of phosphoenzyme in the presence of K^+ was not much affected by washing the membranes.

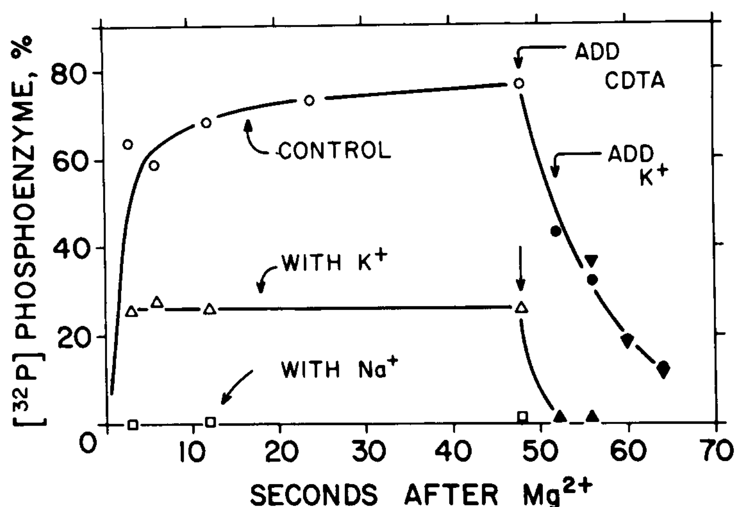

Fig. 9. Phosphorylation from P_i of washed membranes. Membranes were washed in 1 mM $MgCl_2$ at neutral pH for 30–60 min by centrifugation and resuspension. Na^+ and K^+ were present with the enzyme before P_i was added except in the control. $[P_i]$ = 1 mM, $[Mg^{2+}]$ = 2 mM, $[Na^+]$ = $[K^+]$ = 16 mM, [CDTA] = 20 mM. Phosphoenzyme is expressed as percent of the maximal value from $[^{32}P]ATP$ under standard conditions. Reproduced from reference 4.

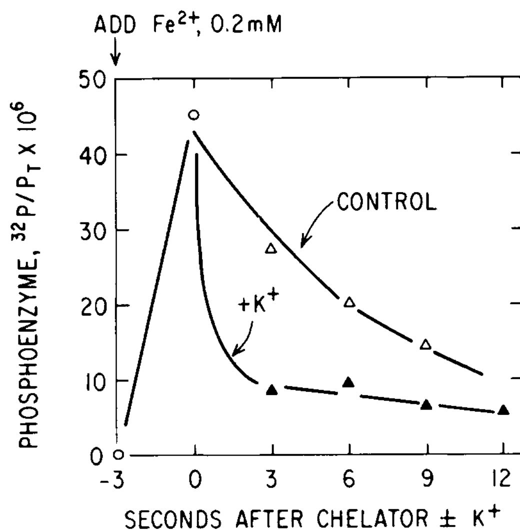

Fig. 10. Formation of K^+-sensitive phosphoenzyme directly from P_i with Fe^{2+} in place of Mg^{2+}. The initial mixture contained 2.3 mg of protein of washed membranes, 20 μmoles of imidazole glycylglycine, and 0.1 μmole of CDTA in 0.8 ml. At 13 sec before 0 time 1 μmole of $^{32}P_i$ in 0.1 ml was added. At 3 sec before 0 time, 0.3 μmoles of $FeCl_2$ in 0.1 ml were added (○). At 0 time 15 μmoles of $(Tris)_3CDTA$ without (△) or with (▲) 8 μmoles of KCl were added in 0.1 ml.

phosphoenzyme. If Na^+ predominates, sensitive phosphoenzyme can become E_1-P and transfer its phosphate group to ATP. If K^+ predominates, K^+-complexed E_2-P is formed, which is unresponsive to ADP.

Net synthesis of ATP was demonstrated in an experiment in which the amount of [^{32}P]ATP produced was three- to fivefold greater than the amount of unlabeled ATP added as a contaminant of the ADP (6). Addition of unlabeled ATP did not affect the reaction (6). Release of free ATP from the enzyme was shown in experiments in which excess dephosphoenzyme or hexokinase and glucose were added after the addition of ADP (6).

Was a Concentration Gradient of Na^+ Across the Membrane Necessary for Synthesis of ATP?

Suppose that fragments of plasma membranes spontaneously resealed to form tight vesicles. Addition of a high concentration of NaCl to the outside of such a vesicle could produce a concentration gradient of Na^+, at least transiently. Such a gradient could be a source of energy. It seems clear that Na^+ must go on the enzyme at a high concentration in order to synthesize ATP, but is it necessary that it also come off again at a low concentration into a different solution on the opposite face of the membrane?

In order to see if there were vesicles in the membrane preparation, an electron microscope picture was made. It showed membrane fragments with broken edges and also many apparently closed vesicles (Fig. 15). In order to make a functional test, a leak-producing agent, gramicidin, was added to the membranes before synthesis of ATP. It had little effect (6). Another leak-producing agent, Lubrol WX, reduced the rate of dephosphorylation of the phosphoenzyme and the rate of synthesis of ATP but did not affect the ratio of ATP recovered to phosphoenzyme split (6).

A test for a gradient was made in a different way. The site from which Na^+ would come off during the synthesis of ATP is the same site at which it goes on to catalyze phosphorylation of the enzyme from ATP. If there is a delay between the time of addition of Na^+ and the time at which it reaches this active site, there should be retardation in the rate of phosphorylation from ATP upon addition of a rate-limiting concentration of Na^+. An experiment with this membrane preparation showed no retardation (6). Therefore, Na^+ could get as easily to the high-affinity site at which it catalyzes the forward reaction as to the low-affinity site at which it catalyzes the reverse reaction. (The $K_{0.5}$ for Na^+ for phosphorylation from ATP is about 1 mM).

Three tests for a gradient failed to produce positive results. It is more difficult to be confident of the absence of a gradient than of its presence, but at present it seems reasonable to assume that there was no gradient and that a gradient is not necessary.

(An estimation of [^{3}H]-inulin space was not made. This estimation would permit calculation of the fraction of the volume of the membrane suspension into which inulin does not penetrate. Even if inulin equilibrated with all the water in the suspension, this information would not help to demonstrate sufficiently rapid penetration of Na^+ into the same space to abolish a concentration gradient of Na^+ during the time course of these experiments. If inulin did not equilibrate with all the water, the demonstration of some tight vesicles would still leave unknown the amount of Na, K-ATPase in the walls of those vesicles.)

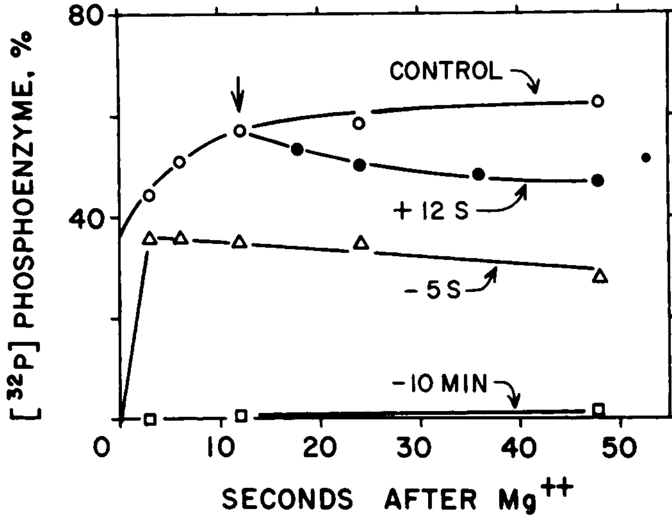

Fig. 11. Slow inhibition by Na^+ of rapid component of phosphorylation from P_i of washed membranes. P_i at 1 mM was added to washed membranes in 0.1 mM CDTA 5 sec before the reaction was started by addition of 2 mM $MgCl_2$. NaCl at 16 mM was added at the times shown before (–) or after (+) 0 time.

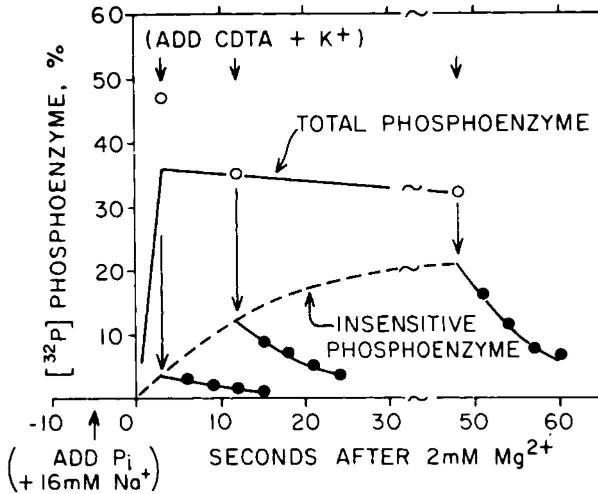

Fig. 12. Stability of K^+-sensitive phosphoenzyme formed from P_i with Mg^{2+} in the presence of Na^+ added just before phosphorylation. To washed membranes in 0.1 mM CDTA was added 1 mM $^{32}P_i$ simultaneously with 16 mM NaCl. Phosphorylation was started 5 sec later with 2 mM $MgCl_2$ at 0 time. At 3, 12, or 48 sec, 18 mM CDTA with 15 mM KCl was added to phosphoenzyme (○) as shown by the arrows (↓) in order to estimate the amount of insensitive phosphoenzyme (●).

DISCUSSION

Interaction Free Energy of Ligand Binding in the Synthesis of ATP

In order to think about a relationship between binding of Na^+ and the energy level of the phosphoenzyme, consider a simple model first (11). Let a protein have two ligands, A and B, and bind one molecule of each, separately or together. For the separate complexes let the dissociation constants be K_a and K_b respectively. Let there be an interaction between the ligands so that the dissociation constant for A to bind to the free protein K_a, is different from that for A to bind to the complex of the protein with B, namely K_{ab}. That is $K_a \neq K_{ab}$. Writing out the equilibria shows that $K_a/K_{ab} = K_b/K_{ba}$ (Eq. 1). That is, whatever the change in the dissociation constant for A produced by the binding of B, there is a corresponding change in the dissociation constant for B produced by the binding of A. This relationship can be expressed more elegantly as an interaction free energy of ligand binding (11). The free energy of dissociation is $\Delta G^\circ = -RT \ln K$ (Eq. 2). From this equation the free energy under standard conditions can be defined for each reaction in the equilibria above. After substitution of the appropriate varieties of Eq. 2 into Eq. 1, the following relationship emerges: $\Delta G_a^\circ - \Delta G_{ab}^\circ = \Delta G_b^\circ - \Delta G_{ba}^\circ = \Delta G_i^\circ$, where ΔG_i° is the interaction free energy. This is the same for the effect of B on A as for the effect of A on B.

Application of these considerations to the synthesis of ATP by Na, K-ATPase leads to the idea that the interaction free energy for the effect of phosphorylation on the binding of Na^+ should be the same as that for the effect of the binding of Na^+ on the free energy of hydrolysis of the phosphate group of the enzyme. At this stage in the investigation the data are so scanty and so indirect that there is no question of testing the hypothesis quantitatively. The question is only whether the data can be maneuvered into tolerating the hypothesis (6).

For dissociation of Na^+ from the phosphoenzyme, the $K_{0.5}$ is about 0.6 M at 0°C according to Fig. 14, giving a ΔG° of +0.8 kcal per 3 Na^+ (compare Fig. 1 for the stoichiometry). For dissociation of Na^+ from the dephosphoenzyme, the $K_{0.5}$ is about 0.6 mM, as judged from the half-maximal concentration for inhibition of phosphorylation from P_i (10). The corresponding ΔG° is + 12 kcal per 3 Na^+. The interaction free energy is therefore about 11 kcal per 3 Na^+. Phosphorylation from P_i is half-maximal at about 0.1 mM for insensitive phosphoenzyme (unpublished experiments). The free energy of hydrolysis of the phosphate group is therefore about +5 kcal/mol. The free energy of hydrolysis of E_1-P can be estimated in a partially poisoned form of the enzyme in which it is stable. The ratio of the concentration of ADP to that of ATP at half-maximal phosphorylation was 10 (10). Consequently, the magnitude of the free energy of hydrolysis is probably somewhat less than that of the terminal phosphate group of ATP, namely about −6 kcal/mole. The interaction free energy is therefore again about 11 kcal per mole. These relationships are illustrated in Fig. 16. It is thus possible to consider interaction free energy of ligand binding as a possible feature of the mechanism of Na, K-ATPase.

Sidedness of the Actions of Na^+

In the scheme of Fig. 16 the dephosphoenzyme has a much greater affinity for Na^+ ($K_{0.5}$ = 0.6 mM) than does the phosphoenzyme ($K_{0.5}$ = 0.6 M). In broken membrane

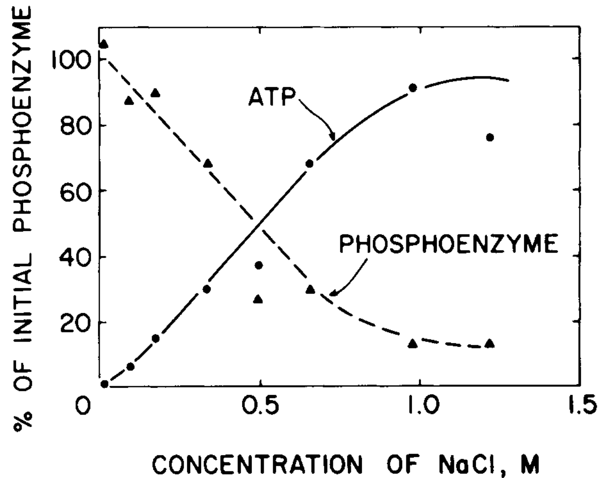

Fig. 13. Synthesis of ATP from K^+-sensitive phosphoenzyme. In a volume of 0.25 ml, 3.9 mg of washed membrane protein was incubated with 5 μmoles of imidazole glyclyglycine, 10 nmoles of $(Tris)_3CDTA$, and 0.5 μmoles of $^{32}P_i$ at pH 7.5 and 0°C. To start phosphorylation, 0.25 μmoles of $MgCl_2$ and 8 μmoles of NaCl were added in 0.05 ml. To start ATP synthesis 4 sec later, 10 μmoles of $(Tris)_3CDTA$ and 0.5 μmole of ADP with various quantities of NaCl were added in 0.2 ml to produce the final concentrations indicated on the horizontal axis. The reaction was stopped with acid 2 sec later to obtain the data shown. Control points were also taken 80 sec later at concentrations of Na^+ at or above 176 mM. At this time no phosphoenzyme remained and the amount of $[^{32}P]$ATP was between 51% and 95% of the initial amount of phosphoenzyme, which was 212 pmoles. The yield of ATP increased progressively with the concentration of Na^+. Reproduced from reference 15.

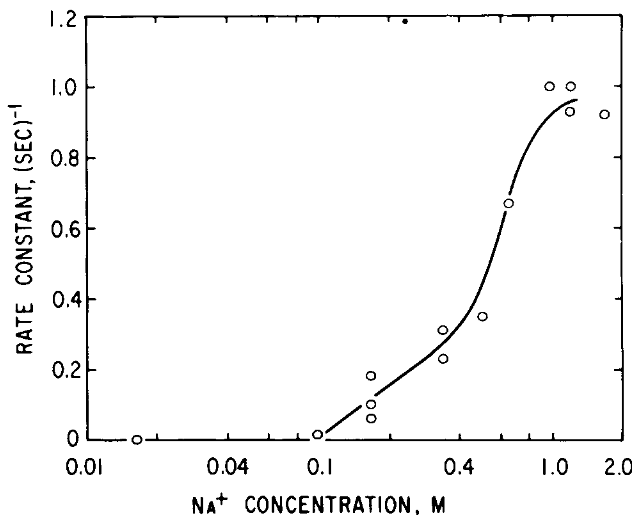

Fig. 14. Apparent monomolecular rate constant for transphosphorylation from K^+-sensitive phosphoenzyme to ADP as a function of Na^+ concentration. Reproduced from reference 6.

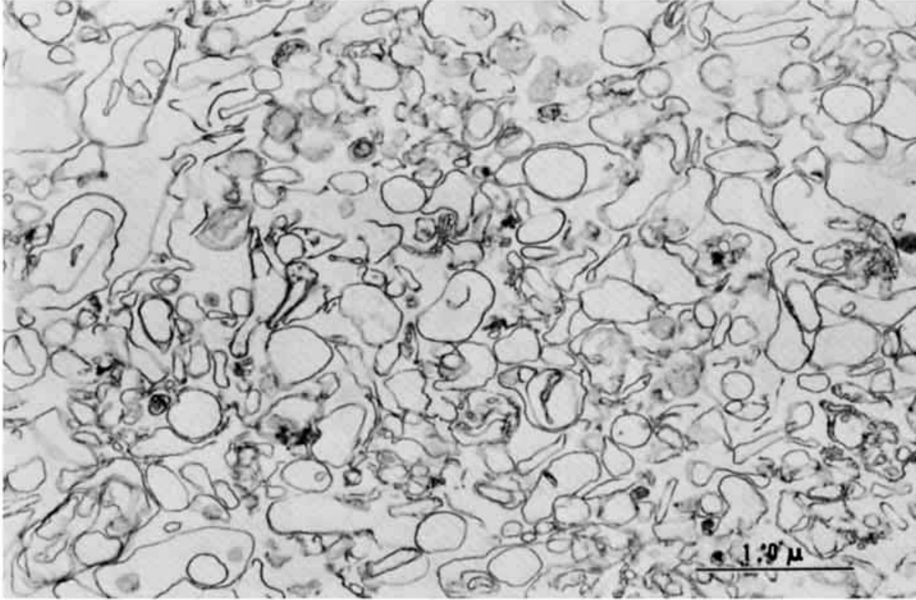

Fig. 15. Electron micrograph of the membrane preparation used in the synthesis of ATP. This photograph was provided through the courtesy of Dr. Sidney Fleischer.

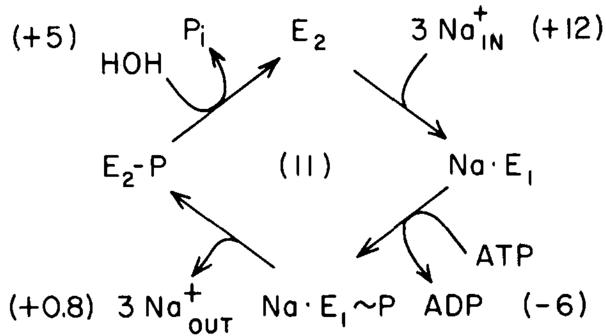

Fig. 16. Scheme of possible free energies of ligand binding of Na^+ and the phosphate group on the enzyme in Na, K-transport ATPase. The assignment of the high affinity site to intracellular Na^+ and the low affinity site to extracellular Na^+ is discussed in the text. The stoichiometry is that of Fig. 1. The symbols are those of Fig. 2. The numbers in parentheses in the corners are estimates of the free energy of dissociation of Na^+ or of hydrolysis of the phosphate group in kcal/mole at 0°C. The number in the center is the interaction free energy. Details are given in the text and in (6). Reproduced from reference 17.

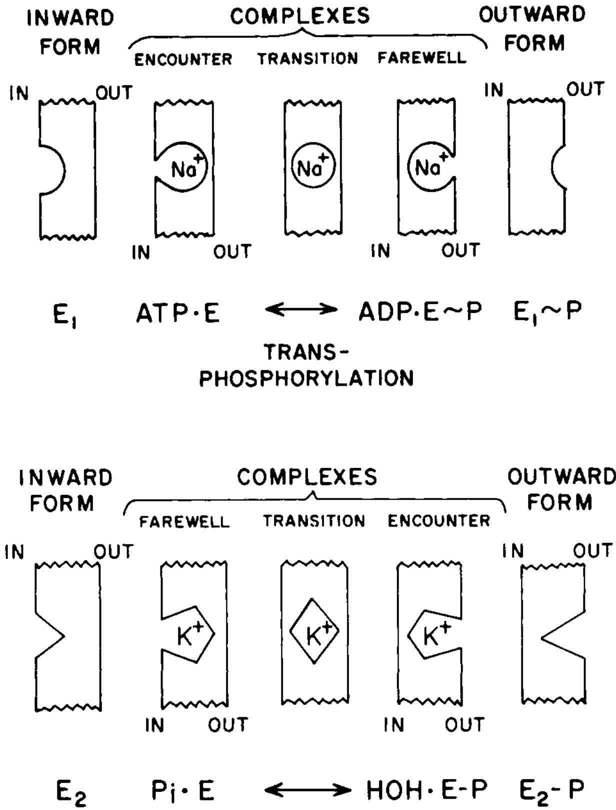

Fig. 17. Diagram of a hypothesis that transition states for ion translocation and those for corresponding ion-activated transphosphorylation reactions are the same. Reactions with respect to Na^+ are in the upper panel and reactions with respect to K^+ are in the lower panel. The implied transition from E_2 to E_1 is probably not by way of the free enzyme as the diagram suggests (compare Fig. 2). Stoichiometry is not specified and Mg^{2+} is not shown. Reproduced from (17). A simpler version of this hypothesis is given by Baker et al. in Fig. 15C of reference 18.

preparations, it is likely that the pump itself retains its sidedness even though the vesicles in which it is located are too leaky to retain concentration gradients or any evidence of transport activity. Evidence for retention of sidedness comes from recent reconstitution experiments in which purified Na, K-ATPase showed transport activity after reconstitution into tight vesicles (12, 13). The high- and low-affinity sites can be assigned to the inner and outer faces of the membrane, respectively, to correspond to the relative affinities of the Na, K-pump for intracellular and extracellular Na^+. The best estimate of the affinities is probably found under conditions of reversible in $\rightleftharpoons$ out exchange of Na^+. This exchange requires the absence of extracellular K^+ and the presence of both ATP and ADP intracellularly (1). Under these conditions, at 37°C the apparent constant for dissociation of Na^+ outside is about 160-fold larger than that for Na^+ inside in human erythrocytes

(14). See reference 1 for references to corresponding data in giant axons and muscle. These conditions probably provide concentrations near to equilibrium for three steps in the scheme of Fig. 16 with hydrolysis of the phosphoenzyme as the rate-limiting step due to the absence of extracellular K^+.

It is therefore reasonable at this stage to assign the high affinity site to intracellular Na^+ and the low affinity site to extracellular Na^+. This assignment implies in turn that phosphorylation controls the sidedness of accessibility of Na^+ to the active site at which it is catalytically active. According to this hypothesis, only intracellular Na^+ has access to the active site in the dephosphoenzyme and only extracellular Na^+ has access in the phosphoenzyme. It is assumed here that the Na^+ which catalyzes transphosphorylation in the ATPase is the same Na^+ which experiences translocation by the pump. This assumption is based on the ionic specificity of the partial reactions of this system. In these Na^+ is unique. On the other hand, K^+ has congeners, Li^+, $NH_4{}^+$, Rb^+, Cs^+, and Tl^+ (although Li^+ occasionally fails to qualify for membership in certain respects) (3).

The control of sidedness by phosphorylation suggests further that transfer of the phosphate group and translocation of monovalent cations share a common transition state. This hypothesis is illustrated in Fig. 17. A nice feature of the hypothesis is that it is as applicable to K^+ as to Na^+.

ACKNOWLEDGMENTS

This research was supported by a grant from the National Heart and Lung Institute, 5R01 HL-01974, and from the National Institute of Arthritis and Metabolic Diseases, 5P01 AM-07462, of the National Institutes of Health.

REFERENCES

1. Glynn, I. M., and Karlish, S. J. D., Annu. Rev. Physiol. 37:13 (1975).
2. Garrahan, P. J., and Glynn, I. M., J. Physiol. 192:237 (1967).
3. Hokin, L. E., and Dahl, J. L., in "Metabolic Pathways," L. E. Hokin, (Ed.). 3rd edition, vol. 6, p. 269, Academic Press, Inc., New York (1972).
4. Post, R. L., Toda, G., and Rogers, F. N., J. Biol. Chem. 250:691 (1975).
5. Skou, J. C., Q. Rev. Biophys. 7:401 (1975).
6. Taniguchi, K., and Post, R. L., J. Biol. Chem. 250:3010 (1975).
7. Post, R. L., Taniguchi, K., and Toda, G., Ann. N.Y. Acad. Sci. 272:80 (1974).
8. Klodos, I., and Skou, J. C., Biochim. Biophys. Acta, 391:474 (1975).
9. Post, R. L., Kume, S., Tobin, T., Orcutt, B., and Sen, A. K., J. Gen. Physiol. 54:306s (1969).
10. Post, R. L., Kume, S., and Rogers, F. N., in "Mechanisms in Bioenergetics," G. F. Azzone, L. Ernster, S. Papa, E. Quagliariello, and N. Siliprandi (Eds.). Academic Press, New York, p. 203 (1973).
11. Weber, G., Ann. N.Y. Acad. Sci. 227:486 (1974).
12. Sweadner, K. J., and Goldin, S. M., J. Biol. Chem. 250:4022 (1975).
13. Hilden, S. H., Rhee, M., and Hokin, L. E., J. Biol. Chem. 249:7432 (1974).
14. Garay, R. P., and Garrahan, P. J., J. Physiol. 231:297 (1973).
15. Post, R. L., Taniguchi, K., and Toda, G., in "Proceedings of the 9th Meeting of the Federation of European Biochemical Societies, Biomembranes, Structure and Function," Akadémiai Kiadó, Budapest, and North Holland Publishing Co., Amsterdam and London 35:231 (1975).
16. Post, R. L., in "Regulatory Functions of Biological Membranes," J. Järnefelt (Ed.). American Elsevier Publishing Co., New York, p. 163 (1968).

17. Post, R. L., Taniguchi, K., and Toda, G., in "Proceedings of a Conference: The Molecular Aspects of Membrane Structure," Battelle Seattle Research Center, November, 1974. Springer Verlag (1975).
18. Baker, P. F., Blaustein, M. P., Keynes, R. D., Manil, J., Shaw, T. I., and Steinhardt, R. A., J. Physiol. 200:459 (1969).