Synthesis of Adenosine Triphosphate and Exchange between Inorganic Phosphate and Adenosine Triphosphate in Sodium and Potassium Ion Transport Adenosine Triphosphatase*

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SUMMARY
Radioactive adenosine triphosphate was synthesized transiently from adenosine diphosphate and radioactive inorganic phosphate by sodium and potassium adenosine triphosphatase from guinea pig kidney. In a first step, K+-sensitive phosphoenzyme was formed from radioactive inorganic phosphate in the presence of magnesium ion and 16 mM sodium ion. In a second step the addition to the phosphoenzyme of adenosine diphosphate with a higher concentration of sodium ion produced adenosine triphosphate. Recovery of adenosine triphosphate from the phosphoenzyme was 10 to 100% in the presence of 96 to 1200 mM sodium ion, respectively. Potassium ion (16 mM) inhibited synthesis if added before or simultaneously with the high concentration of sodium ion but had no effect afterward. The half-maximal concentration for adenosine diphosphate was about 12 μM. Ouabain inhibited synthesis. The ionophore gramicidin had no significant effect on the level of phosphoenzyme nor on the rate nor on the extent of synthesis of adenosine triphosphate. The detergent Lubrol WX reduced the rate of phosphoenzyme breakdown and the rate of synthesis but did not affect the final recovery. Phospholipase A treatment inhibited synthesis. In a steady state, the enzyme catalyzed a slow ouabain-sensitive incorporation of inorganic phosphate into adenosine triphosphate. These results and others suggest that binding of sodium ion to a low affinity site on phosphoenzyme formed from inorganic phosphate is sufficient to induce a conformational change in the active center which permits transfer of the phosphate group to adenosine diphosphate.

Reversal of the ATP-dependent (Na+,K+)-pump has been shown in human red blood cells or their resealed ghosts (1-4). Incorporation of Pi into ATP required concentration gradients of Na+ and K+ across the plasma membrane even higher than those found in vivo. Synthesis was related to reversed net transport of Na+ inward and K+ outward through the pump. Sodium plus potassium ion-dependent adenosine triphosphatase, (Na+,K+)-ATPase, is an activity of the pump in preparations of broken membranes across which concentration gradients cannot develop. In principle, accumulation of concentration gradients across tight membranes impairs experimental estimation of (Na+,K+)-ATPase since some substrates are denied access to their active sites or some products are denied access to most of the experimental medium by the impermeability of the walls of resealed vesicles. Synthesis of ATP by a preparation of (Na+,K+)-ATPase in broken or leaky membranes therefore offers an opportunity to test the role of ion binding as distinguished from ion translocation in the reaction mechanism. The kinetics of the phosphoenzyme of (Na+,K+)-ATPase in red blood cell membranes is similar to that of the enzyme from other sources (5). These experiments were performed on (Na+,K+)-ATPase in a crude suspension of vesicles from a homogenate of guinea pig kidney.

To help the reader follow the design of these experiments, a working hypothesis of the reaction sequence (6, 7) is presented below (Scheme 1).

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1 The abbreviations used are: (Na+,K+)-ATPase, sodium plus potassium ion transport adenosine triphosphatase; CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid.
In this scheme $E$ designates the enzyme and subscripts 1 and 2 designate forms which reversibly accept phosphate from ATP or $P_i$, respectively. $E_1$~$P$ and $E_2$~$P$ are the corresponding products of these reactions. The superscript $s$ indicates that the phosphoenzyme is sensitive to $K^+$ and easily attacked by it.

In addition to these forms there is also an insensitive phosphoenzyme $E_1$-P, which appears when the enzyme is incubated with $P_i$ and $Mg^{2+}$ in absence of inorganic monovalent cations (8). In earlier experiments from this laboratory insensitive phosphoenzyme was the starting material for synthesis of ATP (9).

The experiments presented here report synthesis of ATP from sensitive phosphoenzyme, $E_1$-P, prepared directly from $P_i$ and compare this reaction with that from insensitive phosphoenzyme, $E_2$-P, in the same membrane preparation. In addition, synthesis from insensitive phosphoenzyme is characterized further.

METHOD

The enzyme preparation consisted of crude membranes prepared from a homogenate of guinea pig kidney according to Post and Sen (10). They were treated further with NaI by the method of Nakao et al. (11) according to the modification of Hegyvary and Post (12). The amount of enzyme in a sample was estimated by phosphorylation from ATP with $Na^+$ and $Mg^{2+}$ under standard conditions (8). The membrane fragments were washed briefly with 1 mM MgCl$_2$ by suspension and centrifugation according to Post et al. (8). To synthesize ATP, $K^+$-sensitive or insensitive phosphoenzyme was prepared first and a high concentration of $Na^+$ with ADP and CDTA was added. The reaction was carried out at pH 7.4 and 0° unless otherwise stated. The reaction was stopped by addition of 1 ml of 0.57 M trichloroacetic acid containing 74 μmol of $NaPO_4$ and 1.00 μmol of unlabeled ATP. The denatured membrane suspension was centrifuged at 17,000 $\times$ g for 20 min at 0° and the supernatant was extracted three times with 4 ml of butyl acetate and twice with 4 ml of hexanes at 23°. ATP was isolated by column chromatography at 23° on Dowex AG 1-X4 as described (9) except that the flow rate was reduced to 3.6 ml/hour and fractions were collected every 55 min. Also the concentration of Trias-HCl (pH 2.5) in the third and fourth chambers of the gradient generator was increased to 150 from 30 mM. The amount of $[^32]P$ATP synthesized was estimated from the ratio of the product of the specific activity of the $[^32]P$, the extinction coefficient for ATP at pH 2.5 (namely 15.5), and the "difference" in the counts per min per ml to the "difference" in the absorbance at 260 nm. The "difference" refers to the differences between values obtained in the reaction containing the maximum absorbance at 260 nm and those in the second following fraction.

The amount of $[^32]P$phosphoenzyme in the precipitate of the denatured membranes was estimated by peptic digestion and paper electrophoresis of the active site phosphopeptides as described (8). In order to correct for losses due to hydrolysis of the labile phosphate bond during this procedure the same lot of enzyme was phosphorylated from $[^32]P$ATP under standard conditions (13) and aliquots were taken for estimation of the phosphoenzyme both by Millipore filtration and by peptic digestion and paper electrophoresis (8). The value by Millipore filtration was taken as 100%. The loss during peptic digestion and paper electrophoresis was between 40 and 70%. Protein was estimated by the method of Lowry et al. (14) with bovine serum albumin as a standard.

RESULTS

Effect of $Na^+$ Concentration on Synthesis of ATP from $K^+$-sensitive or Insensitive Phosphoenzyme—In earlier experiments from this laboratory (9), the starting material was insensitive phosphoenzyme, $E_1$-P. Evidence was presented that 160 mM NaCl slowly converts insensitive phosphoenzyme into sensitive phosphoenzyme and the inference was made that sensitive phosphoenzyme was an intermediate in the synthesis of ATP. In order to test directly the effectiveness of sensitive phosphoenzyme as a substrate for ATP synthesis, it was prepared directly from $P_i$.

The experimental basis for the direct preparation of sensitive phosphoenzyme from $P_i$ will be presented elsewhere. The procedure includes the following steps, (a) washing with 1 mM MgCl$_2$, (b) removal of free $Mg^{2+}$, and (c) addition of $P_i$, and (d) addition of MgCl$_2$ simultaneously with a low concentration of NaCl (4 to 16 mM). The resulting sensitive phosphoenzyme slowly becomes insensitive phosphoenzyme during a period of about 30 s at 0°.

A high concentration of NaCl was prerequisite for the synthesis of ATP from insensitive phosphoenzyme (9). This requirement was investigated for $K^+$-sensitive phosphoenzyme. It was formed and synthesis was started by addition of ADP and CDTA with various concentrations of NaCl. After 2 or 80 s the reaction was stopped and phosphoenzyme and ATP were measured. Increase of NaCl concentration decreased remarkably the amount of phosphoenzyme at 2 s. The amount of ATP synthesized during 2 s increased correspondingly and became saturated at about 1.2 mM NaCl. After 80 s phosphoenzyme disappeared almost completely, except that in the presence of 16 or 96 mM NaCl about 14 or 12% of phosphoenzyme remained, respectively. In the presence of the highest concentrations of NaCl each mole of phosphoenzyme was almost completely converted to 1 mol of ATP (Fig. 1).

In another experiment in which sensitive phosphoenzyme was made with 0.4 mM MgCl$_2$ and 16 mM NaCl the results were approximately the same.

To compare the synthesis of ATP from $K^+$-sensitive phosphoenzyme with that from insensitive phosphoenzyme in the same enzyme preparation, the enzyme was incubated with $P_i$ and 1 umol $Mg^{2+}$ for a longer time to form insensitive phosphoenzyme. Insensitive phosphoenzyme was less responsive to NaCl than $K^+$-sensitive phosphoenzyme and the rate of synthesis of ATP was increased.

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1 G. Toda and R. L. Post, manuscript in preparation.
amol of NaCl was added to start the synthesis of ATP. At 110 a,
 enzyme was estimated at 30 s after initiation of phosphorylation.
-01 of (Tris)&DTA was added. After the time intervals indi-
synthesized ATP. Phosphorylation was started by addition of 0.01
containing 1.15 mg of membrane protein, 5 amol of imidazole
and various quantities of ADP to produce the concentrations indicated. At 160 s the reaction
In most experiments CDTA was used to chelate free MgCl to interrupt phosphorylation
and without ( ) or without ( ) 10
of (Tris)CDTA was added. After the time intervals indicated, the reaction was stopped. The initial amount of phosphoenzyme was estimated at 30 s after initiation of phosphorylation.
Fig. 5 (center). Breakdown by excess (Na+, K+)-ATPase of synthesized ATP. Phosphorylation was started by addition of 0.01
ml containing 0.125 amol of MgCl to 0.22 ml of a reaction mixture containing 1.15 mg of membrane protein, 5 amol of imidazole
, 0.026 amol of (Tris)CDTA, and 0.36 amol of 31P; at zero time. At 30 s, 0.02 ml containing 0.25 amol of ADP and 40
of NaCl was added to start the synthesis of ATP. At 110 s, 0.5 ml containing 0.2 mg of MgCl, 80 amol of NaCl, 10 amol of
imidazole glycylglycine, 0.5 amol of unlabeled P, and 0.5 amol of ADP with ( ) or without ( ) 4.07 mg of membrane protein was added. After the time intervals indicated the reaction was stopped. The amount of initial phosphoenzyme was estimated from the amount of phosphoenzyme at 30 s. EP indicates phosphoenzyme.
Fig. 0 (right). The effect of concentration of ADP on synthesis of ATP. Phosphorylation was started by addition of 0.05 ml containing 0.25 amol of MgCl to 0.25 ml of a reaction mixture containing 2.97 mg of membrane protein, 5 amol of (Tris)CDTA, and 6.5 amol of 31P. At zero time. At 120 s synthesis of ATP was started by addition of 0.2 ml containing 625
muol of NaCl, 10 amol of (Tris)CDTA, and various quantities of ADP to produce the concentrations indicated. At 160 s the reaction was stopped by acid and 31P]ATP ( ) was estimated. The final concentration of unlabeled ATP in the commercial ADP preparation was 0.6% of the ADP concentration ( ). One hundred per-
ent of phosphoenzyme was estimated from the amount at 120 s. If ADP does not affect the spontaneous hydrolysis of phosphoenzyme, the rate constant of hydrolysis and the rate constant of ATP synthesis are calculated to be 0.03 ( ) and 0.12 ( ) respectively. Application of these values to the data in Fig. 6 gives a half-
maximal concentration of ADP on the apparent rate constant of ATP synthesis of 12 μM.
crease of ATP was not observed. In another experiment Mg$^{2+}$ was added with the ADP so that the final concentration of free Mg$^{2+}$ was 1.2 mM. In this case all ATP disappeared within 40 s. Ouabain strongly inhibited synthesis of ATP in the presence of CDTA (9). To investigate the effect of ouabain in the absence of CDTA, enzyme was incubated with ouabain, P$_i$, and Mg$^{2+}$ to form ouabain-phosphoenzyme. After 30 s, Na$^+$ with ADP was added and the reaction was stopped with acid. The presence of ouabain increased the amount of phosphoenzyme about 1.8-fold and stabilized it. ATP was not synthesized significantly (Table I). This result shows that ouabain-insensitive varieties of ATP synthetase were not active in this system.

**Table I**

<table>
<thead>
<tr>
<th>Time after sodium with ADP</th>
<th>Phosphoenzyme</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>160</td>
<td>101</td>
<td>1.2</td>
</tr>
<tr>
<td>320</td>
<td>102</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Effect of ADP Concentration on Synthesis of ATP**—In order to investigate the effect of ADP concentration on synthesis of ATP, 1.25 mM Na$^+$ and CDTA with various concentrations of ADP were added to insensitive phosphoenzyme. The synthesis was stopped after 40 s. The amount of ATP synthesized was nearly half the amount of phosphoenzyme present in the initial mixture and 10 μmol of CDTA were not added. From this experiment it was appropriate to investigate the effect of excess unlabeled ATP on synthesis of $[^{32}P]$ATP. The synthesis was started by addition of 0.125 μmol of MgCl$_2$ with 1 μmol of KCl to 0.22 ml of a mixture containing 3.04 mg of membrane protein, 5 μmol of imidazole glycylglycine, 0.025 μmol of (Tris)CDTA, and 0.25 μmol of $[^{32}P]$Pi at pH 7.5 and 0°C or 22°C. At 5 s, 0.02 ml containing 0.25 μmol of ADP and 5 μmol of CDTA with or without 40 μmol of NaCl was added. The reaction was stopped at 26 s by acid. The amount of $[^{32}P]$phosphoenzyme at 0 s was taken as 100%. The values in parentheses were obtained at 22°C; those outside were obtained at 0°C.

**Table II**

<table>
<thead>
<tr>
<th>NaCl</th>
<th>Phosphoenzyme</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5 (1.4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>+</td>
<td>2.3 (1.4)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

**Effect of ATP on Synthesis of ATP**—Since ATP is a product of the reaction, it was appropriate to investigate the effect of excess unlabeled ATP on synthesis of $[^{32}P]$ATP. The synthesis was started by addition of 1.25 mM Na$^+$, 0.1 mM ADP, and CDTA with various concentrations of unlabeled ATP to insensitive phosphoenzyme. The reaction was stopped after 40 s and $[^{32}P]$phosphoenzyme and $[^{32}P]$ATP were measured. At concentrations of 2, 10, 40, 120, 1800, and 5000 μM ATP, no detectable effect was observed. No detectable phosphoenzyme remained after the reaction was stopped.**

**Lack of ATP synthesis from potassium-complexed phosphoenzyme** (Mg$^-$K$^+$E$_2$P)—In order to investigate synthesis of ATP from potassium-complexed phosphoenzyme (8), the enzyme was incubated with $P_i$, Mg$^{2+}$, and K$^+$ at 0 or 22°C. ADP and CDTA were added at 0 or 23°C. The reaction was stopped at 26 s by acid. The amount of Mg$^-$K$^+$E$_2$P at 6 s at 0°C and at 22°C was about 13 or 21%, respectively, of the amount of enzyme. At 26 s almost all of the phosphoenzyme had disappeared but ATP was not synthesized (Table II). Another experiment with a 40-fold higher concentration of K$^+$ also did not produce any ATP.

**Effect of K$^+$ on Synthesis of ATP**—K$^+$ and congeners of K$^+$ partially inhibited synthesis of ATP when they were added at low concentrations simultaneously with Na$^+$, ADP, and CDTA (9). When 17 mM KCl was added with 0.68 mM NaCl, 1 mM ADP, and 20 mM (Tris)CDTA to sensitive phosphoenzyme the maximal recovery of ATP 80 s later was less than 2% of the amount of phosphoenzyme. There are at least two possible mechanisms for inhibition. One is that K$^+$ forms K$^+$-complexed phosphoenzyme (Mg$^-$K$^+$E$_2$P) more rapidly than concentrated Na$^+$ converts Mg$^-$E$_2$P into ADP-sensitive phosphoenzyme (Mg$^-$Na$^+$E$_2$P). Another mechanism is that K$^+$ reacts directly with Mg$^-$Na$^+$E$_2$P to inhibit its reaction with ADP. In order to distinguish between these possibilities, a high concentration of Na$^+$ was added to sensitive phosphoenzyme in order to permit its conversion into Mg$^-$Na$^+$E$_2$P. Two seconds later, the reaction mixture was diluted 21-fold to reduce the Na$^+$ concentration enough to prevent further conversion of Mg$^-$E$_2$P into Mg$^-$Na$^+$E$_2$P, as shown by a preliminary experiment. The diluted contained ADP and CDTA with or without K$^+$. After this 2-s pulse of a high concentration of Na$^+$ and at the beginning of incubation with ADP and CDTA, about half of the initial phos-
The phosphorylation reaction was started by addition of 0.01 ml containing 0.125 μmol of MgCl₂ with 4 μmol of NaCl to 0.125 ml of a reaction mixture containing 1.7 mg of membrane protein, 5 μmol of imidazole glycylglycine, 0.025 μmol of (Tris)₂CDTA, and 0.25 μmol of [³²P] ATP, at zero time. After 4 s, 0.1 ml containing 160 μmol of NaCl was added with or without 83 μmol of KCl. At 6 s, the reaction mixture was diluted 21.3-fold with 4.77 ml containing 5 μmol of ADP* and 100 μmol of (Tris)₂CDTA without or with 83 μmol of KCl, respectively. At the indicated times after dilution the reaction was stopped with acid and the amounts of phosphoenzyme and ATP were estimated. The amount of phosphoenzyme at 6 s was taken as 100% and all quantities are expressed as per cent of this value. EP = phosphoenzyme.

<table>
<thead>
<tr>
<th>Time</th>
<th>Omit K⁺</th>
<th>K⁺ with ADP</th>
<th>K⁺ with 0.68 mM Na⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EP</td>
<td>ATP</td>
<td>EP</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>23</td>
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<td>3</td>
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<td>7</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
<td>22</td>
<td>1.6</td>
</tr>
<tr>
<td>8</td>
<td>52</td>
<td>29</td>
<td>1.6</td>
</tr>
<tr>
<td>10</td>
<td>36</td>
<td>26</td>
<td>1.5</td>
</tr>
<tr>
<td>12</td>
<td>50</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

These data and those in Ref. 19 suggest that 16 mM K⁺ attacks Mg·E₀-P more rapidly than 0.68 mM Na⁺ converts it into Mg·Na·E₀-P. They suggest further that concentrations of K⁺ below 16 mM do not inhibit the reaction of Mg·Na·E₀-P with ADP.

**Effect of Oligomycin—Oligomycin is assumed to inhibit the phosphorylation reaction sequence between Mg·Na·E₀-P and Mg·E₀-P (15, 16). In experiments with phosphorylation directly from P₁ oligomycin inhibited Mg·E₀-P formation but not Mg·K·E₀-P formation (8). The effect of oligomycin on synthesis of ATP was tested at 176 mM NaCl. Oligomycin (400 μg/ml) reduced the initial level of sensitive phosphoenzyme formed from P₁ and Mg⁺⁺ with Na⁺ to 60% of the control. It also inhibited ATP synthesis from the phosphoenzyme from 48% in the control to 31% of the reduced amount of phosphoenzyme. The relative inhibition was the same at all times from 5 to 80 s. Synthesis was complete at 30 s (data not shown). Oligomycin appeared to inhibit in the backward direction as well as in the forward direction.

**Effect of Gramicidin on Synthesis of ATP—Gramicidin is an ionophore for transport of monovalent cations through artificial thin lipid membranes and those of red blood cells (17). If a gradient of Na⁺ concentration across the walls of the vesicles present in the enzyme preparations is prerequisite for the synthesis of ATP, synthesis could be inhibited in principle by relatively low concentrations of gramicidin (several micrograms per ml) (17). To investigate this, the enzyme was incubated with P₁ and various concentrations of gramicidin. ATP synthesis from insensitive phosphoenzyme was estimated at high and low concentrations of Na⁺. Gramicidin had little effect (Fig. 7). The rate constant of breakdown of phosphoenzyme in the presence of ADP and CDTA was increased about 10% by gramicidin (20, 660, and 1920 μg/ml) in the presence of 60 or 160 mM Na⁺. In another experiment the enzyme was incubated with P₁ and Mg⁺⁺ with or without gramicidin (600 μg/ml) at 35° and pH 7.5 for 10 min. Phosphorylation and subsequent ATP synthesis were carried out as in Fig. 7 except that the temperature was 25°, and the Na⁺ concentration was only 160 mM. The intervals of synthesis were 5-fold shorter. Gramicidin had no detectable effect at all.

**Effect of Lubrol WX on Synthesis of ATP—Lubrol WX disrupts membrane structure and solubilizes (Na⁺, K⁺)-ATPase (18-21). To explore its action insensitive phosphoenzyme was treated with Na⁺, ADP, and CDTA with various concentrations of Lubrol WX. The reaction was stopped by acid and phosphoenzyme and ATP were estimated. In the presence of about 1 to 2% (v/v) of Lubrol WX, which should be enough to disrupt the membrane (18-21), the rate of breakdown of phosphoenzyme and the synthesis of ATP were reduced (Table IV). But recovery of ATP relative to the disappearance of phosphoenzyme was unaffected. The results suggest that intact membrane structure is not prerequisite for the synthesis of ATP.

**Effect of Preliminary Incubation with Na⁺ on Phosphorylation from ATP—If translocation of Na⁺ across the membrane is prerequisite for ATP synthesis, then Na⁺ must not only bind to the...
enzyme but must also subsequently leave the enzyme. Specifically Na⁺ must dissociate from the site (or sites) at which it catalyzes the forward reaction, namely, transphosphorylation from ATP to the enzyme. In the reversal of translocation Na⁺ would bind to the enzyme on the extracellular face of the membrane and would dissociate from the intracellular face (compare Scheme 1).

If a significant amount of vesicular structure impermeable to Na⁺ is present in the enzyme preparation, the rate of phosphorylation from ATP under conditions in which binding of Na⁺ is rate-limiting should be significantly slower than the rate obtained in which the binding is not rate-limiting. In order to investigate this possibility, the enzyme was incubated without or with Na⁺ at two concentrations for 20 min at 37°C and pH 7.5 and then cooled on ice. [³²P]ATP was added next and 60 s later phosphorylation was started by addition of MgCl₂ with or without Na⁺, respectively. The level of phosphorylation at 0.8 mM Na⁺ was about half of that at 16 mM Na⁺ which is at least 10-fold higher than the Kᵣ. Phosphorylation was almost complete within 3 s at both concentrations regardless of the previous duration of exposure to Na⁺ (Fig. 6). At both concentrations of Na⁺ preliminary incubation with Na⁺ produced a slightly higher level of phosphorylation. This effect might represent renaturation of a partially denatured fraction of the enzyme rather than delayed access of Na⁺ to its active site (or sites) since the effect was not dependent on Na⁺ concentration in this range. The membrane preparation did not show significant impermeability to Na⁺ by this test.

**Effect of Treatment with Phospholipase on Synthesis of ATP**—Phospholipids probably participate not only in phosphorylation from ATP but also in dephosphorylation of the enzyme (22). To investigate the role of phospholipids in the synthesis of ATP, the enzyme was treated with phospholipase A in the presence or absence of CaCl₂ (23). Preparations thus obtained are designated as ATP X 100/(100 - EP). Recovery of ATP relative to the disappearance of phosphoenzyme, EP, was calculated as ATP X 100/(100 - EP).

<table>
<thead>
<tr>
<th>Lubrol WX</th>
<th>EP</th>
<th>ATP</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>% (b/s)</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>1.2</td>
<td>24.0</td>
<td>23.3</td>
</tr>
<tr>
<td>0.96</td>
<td>29.2</td>
<td>17.7</td>
<td>25.0</td>
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<tr>
<td>1.92</td>
<td>31.0</td>
<td>14.7</td>
<td>21.3</td>
</tr>
</tbody>
</table>

**Effect of phospholipase A treatment on phosphoenzyme formation and ATP synthesis**

Membranes containing 24 mg of protein were treated in the presence or absence of CaCl₂ with phospholipase A as reported previously (23) except that fatty acid-poor albumin was used instead of crystalline bovine serum albumin. The samples were diluted 8.5-fold with water and packed by centrifugation (27,000 × g for 45 min). The samples were suspended in a solution containing 10 mM imidazole, 2 mM HCl, and 0.1 mM HEDTA and packed as before. The precipitates were suspended in 1 ml of 10 mM imidazole-HCl buffer containing 0.1 mM HEDTA (pH 7.5). The protein concentrations of control and treated preparations were 13.8 and 11.8 mg/ml, respectively. Phosphorylation from ATP was started by addition of 0.05 ml containing 0.25 μmol of MgCl₂ and 0.045 μmol of [³²P]ATP to 0.45 ml of a reaction mixture containing 0.03 ml of control or treated preparation, 5 μmol of imidazole glycylglycine, and 80 μmol of NaCl or 8 μmol of KCl. After 5 s, the reaction was stopped with acid. Phosphorylation from P₁ was started by addition of 0.05 ml containing 0.25 μmol of MgCl₂ to 0.25 ml of a reaction mixture containing 0.13 ml of control or treated preparation, 5 μmol of imidazole glycylglycine, and 0.5 μmol of [³²P] with or without 0.125 μmol of ouabain. After 180 s the reaction was stopped by acid or the synthesis of ATP was started by addition of 0.05 ml containing 0.25 μmol of MgCl₂ to 0.25 ml of a reaction mixture containing 0.13 ml of control or treated preparation, 5 μmol of imidazole glycylglycine, and 0.5 μmol of [³²P], with or without 0.125 μmol of ouabain. After 180 s the reaction was stopped by acid. The amounts of phosphoenzyme and ATP were estimated. The rate constant of phosphoenzyme breakdown was estimated from the decrease of the phosphoenzyme after starting the ATP synthesis.

**Table IV**

**Effect of Lubrol WX on synthesis of ATP**

Phosphorylation was started by addition of 0.05 ml containing 0.25 μmol of MgCl₂ to 0.25 ml containing 2.3 mg of membrane protein, 5 μmol of imidazole glycylglycine, and 0.05 μmol of [³²P]. At 30 s synthesis of ATP was started by addition of 0.2 ml containing 0.5 μmol of ADP, 80 μmol of NaCl, and 10 μmol of (Tris)₂CDTA with quantities of Lubrol WX to produce the final concentrations indicated. The reaction was stopped at 110 s. The amount of phosphoenzyme obtained at 30 s was taken as 100%. Recovery of ATP relative to the disappearance of phosphoenzyme, EP, was calculated as ATP X 100/(100 - EP).

**Table V**

**Effect of phospholipase A treatment on phosphoenzyme formation and ATP synthesis**

<table>
<thead>
<tr>
<th>Source of³²P</th>
<th>Control</th>
<th>Treated</th>
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</thead>
<tbody>
<tr>
<td>ATP</td>
<td>175</td>
<td>121</td>
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<tr>
<td>Pi</td>
<td>89</td>
<td>23</td>
</tr>
<tr>
<td>Pi + ouabain</td>
<td>222</td>
<td>54</td>
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</tbody>
</table>

**ATP synthesis**

<table>
<thead>
<tr>
<th>Time</th>
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<th>Treated</th>
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<tbody>
<tr>
<td>s</td>
<td>µmol/sample</td>
<td>µmol/sample</td>
</tr>
<tr>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>10</td>
<td>9.8</td>
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<td>20</td>
<td>10.0</td>
<td>1.3</td>
</tr>
<tr>
<td>30</td>
<td>17.6</td>
<td>1.3</td>
</tr>
</tbody>
</table>

| Rate constant of breakdown of phosphoenzyme from Pi₁ | 0.060 | 0.038 |

**P₁ → ATP Exchange**—Incubation of the enzyme with Na⁺ and P₁ followed by addition of Mg²⁺ completely inhibited phosphoenzyme formation from P₁ (8). However, if unlabeled ATP is added with Mg²⁺ in this system, the enzyme accepts phosphate from ATP to form unlabeled phosphoenzyme which exchanges its phosphate group with free P₁ (8). In the presence of [³²P] radioactive Mg₂E₂⁻P appears. If a high enough concentration of Na⁺ is present, enough to convert some Mg₂E₂⁻P back to Mg-Na₁E₂⁻P, [³²P]ATP should appear after addition of ADP with CDTA. To test this possibility enzyme was incubated with high or low concentrations of Na⁺ or K⁺ with [³²P] and the phosphorylation reaction was started by addition of Mg²⁺ and
TABLE VI
Incorporation of $^{32}$P from Pi into ATP in presence of ATP and high concentration of Na$^+$

In the complete system 6.5 mg of membrane protein were incubated with 1 lmol of $^{32}$Pi, 160 lmol of NaCl, and 10 lmol of imidazole glycylglycine in a final volume of 0.5 ml overnight at pH 7.5 and 0$. The reaction was started by addition of 0.1 ml containing 2 lmol of MgCl$_2$ and 0.01 lmol of unlabeled ATP. After 30 s 0.1 ml containing 1 lmol of ADP with 20 lmol of (Tris)CDTA was added. The reaction was stopped by acid at 50 s after starting the reaction. [32P]ATP and $[^{32}$P]phosphoenzyme were estimated. When present, 0.25 lmol of ouabain was added with Mg$^{2+}$ and ATP. In some samples, 160 mM NaCl was replaced by 16 mM NaCl or 160 mM KCl. The amount of phosphoenzyme obtained was 30 s after starting the reaction in the complete system was taken as 100%.

<table>
<thead>
<tr>
<th>Incubation with Na$^+$ (mM)</th>
<th>[32P]Phosphoenzyme</th>
<th>[32P]ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$ = 160</td>
<td>2</td>
<td>18.5</td>
</tr>
<tr>
<td>Na$^+$ = 160 + ouabain</td>
<td>175</td>
<td>0</td>
</tr>
<tr>
<td>Na$^+$ = 160</td>
<td>154</td>
<td>1.9</td>
</tr>
<tr>
<td>K$^+$ = 160</td>
<td>1.9</td>
<td>0</td>
</tr>
</tbody>
</table>

The amount of phosphoenzyme from $^{32}$Pi increased slowly with time, and it showed nearly the same kinetics as the incorporation of $^{32}$Pi into ATP (Fig. 10). At 200 s the amount was almost 0.5%. In the presence of ouabain, the rate of phosphoenzyme formation from $^{32}$Pi was rapid, reaching about 50% of the enzyme at 200 s. Phosphorylation from ATP was most rapid; about 72% of the enzyme was phosphorylated within 10 s.

DISCUSSION
Reversal of (Na$^+$,K$^+$)-ATPase Reaction via Functional Phosphoenzyme—These experiments showed net synthesis of ATP from ADP and Pi. The amount of [32P]ATP recovered was greater than the amount of unlabeled ATP added (Fig. 6). The reaction was insensitive to added ATP (see text). Synthesized ATP was released from the enzyme within a few seconds (Fig. 5 and text).

In a first step, inorganic phosphate was incorporated transiently into the enzyme in the presence of Mg$^{2+}$ at a low concentration of Na$^+$ to form K$^+$-sensitive phosphoenzyme, Mg$^+$E$^+$P, which is also an intermediate from ATP in the forward direction (8). Treatment of the K$^+$-sensitive phosphoenzyme with a high concentration of Na$^+$ developed sensitivity to ADP and synthesis of ATP (Fig. 1). Sensitivity to ADP and insensitivity to K$^+$ persisted momentarily after the high concentration of Na$^+$ was reduced by dilution (Table III). The results indicate that addition of Na$^+$ to Mg$^+$E$^+$P converts it into Mg$^+$Na$^+$.E$^+$P (Scheme 1). Reversibility of this step in a steady state was indicated by a slow ouabain-inhibitable Pi→ATP exchange (Figs. 9, 10, Table VI), which has not been detected previously (24–26).

The functional importance of K$^+$-sensitive phosphoenzyme as a precursor of Mg$^+$Na$^+$.E$^+$P is emphasized by its more rapid rate of reaction, as compared with insensitive phosphoenzyme, which appears in the absence of monovalent inorganic cations (9) (Figs. 2 and 3). Furthermore complexes of the phosphoenzyme with ouabain (Table I) or K$^+$ (Table II) were ineffective substrates for synthesis of ATP. Also oligomycin partially inhibited synthesis (see text); this inhibition is consistent with its assumed inhibition of conversion of E$^+$P→E$^+$P (15, 16). Synthesis of ATP did not appear to require free Mg$^{2+}$ as if the Mg$^{2+}$ required for the initial phosphorylation remained bound to the phosphoenzyme (8). Synthesis was remarkably sensitive to ADP with a $K_m$ about 12 $\mu$M. This value is higher than the dissociation constant from the dephosphoenzyme in the presence of Na$^+$, 0.34 $\mu$M (27) or in the absence of Na$^+$, 2 $\mu$M (12) or 1 $\mu$M (28).

Role of Phospholipids in Synthesis of ATP—Phospholipase A treatment markedly decreased phosphorylation from Pi$^-$, with or without ouabain, and only moderately decreased phosphorylation from ATP. The largest effect was the almost complete in-
hibition of ATP synthesis (Table V). Taniguchi and Tonomura have shown that phosphoenzyme formed from ATP after treatment with phospholipase A was K⁺-insensitive (22). This treatment also removes a discontinuity in the activation energy of the ATPase activity (23). These data suggest that phospholipids are important for a conformational change between ADP-sensitive phosphoenzyme and K⁺-sensitive phosphoenzyme.

Is Translocation of Na⁺ Required for Synthesis of ATP?—The most striking feature of these synthesis experiments was the requirement for a high concentration of Na⁺. The half-maximal concentration was about 0.6 M (Fig. 3). This contrasts with the half-maximal concentration of Na⁺ required for phosphorylation from ATP in the forward direction, 0.3 to 1.6 mM in our experience (29). With respect to reversibility in exchange of Na⁺ through the pump in human erythrocytes, Garay and Gharahan (30) have estimated half-maximal concentrations about 119 mM for extracellular Na⁺ and about 0.7 mM for intracellular Na⁺. (Note. These authors gave values for dissociation constants from a three-site model. We have converted their values to estimates of Kₐₜ by multiplying them by the reciprocal of (21/ₜ - 1). These differences in concentration suggest that the Na⁺ which catalyzes phosphorylation from ATP is combining with the translocation site for Na⁺ when it is in communication with the medium in contact with the intracellular face of the membrane, or what was the intracellular face of the membrane in the intact cell. Similarly the Na⁺ which catalyzes ATP synthesis may be combining with its translocation site when that site is in communication with the medium in contact with the extracellular face of the membrane. (These assumptions are consistent with a model in which the translocation site faces inward in the dephosphoenzyme and outward in the phosphoenzyme (31).) Accordingly the Na⁺ which catalyzes ATP synthesis binds to the enzyme on the extracellular face of the membrane. The question for discussion is whether this binding is sufficient or is it also necessary that Na⁺ subsequently dissociate from the pump at the intracellular face of the membrane. That is, is translocation of Na⁺ necessary for synthesis of ATP?

(Na⁺,K⁺)-ATPase is an intrinsic protein embedded in the plasma membrane with access to both faces (32) as one might infer from the sidedness of the reaction (15, 16). In tissue homogenates plasma membrane fragments spontaneously reseal to form vesicles. In our preparation were the vesicles sufficiently tight to sustain a transient concentration gradient of Na⁺ or not? It is difficult to be confident of the absence of a gradient, particularly in a crude and homogeneous preparation, but the following considerations support such a conclusion. (a) A concentration of Na⁺ which is rate limiting for the forward reaction was almost equally effective whether it was added to the reaction mixture early or late (Fig. 8). (b) The inophore gramicidin had no significant effect (Fig. 7 and text). (c) The detergent Lubrol WX stabilized the phosphoenzyme but did not affect the yield of ATP relative to the loss of phosphoenzyme (Table IV). (d) Release of Na⁺ cannot precede transphosphorylation in the backward reaction since binding of Na⁺ is required for transphosphorylation in the forward reaction. Release of Na⁺ probably has little effect on release of newly synthesized but still bound ATP since Na⁺ has little effect on the affinity of the dephosphoenzyme for ATP in the absence of Mg²⁺ (12). In conclusion, it seems likely that binding of Na⁺ was sufficient for synthesis of ATP and that a subsequent step releasing Na⁺ (from the opposite face of the membrane) was not necessary.

Where Does the Energy Come From?—Since binding of Na⁺ seems to be sufficient to convert the phosphoenzyme from a form equilibrating with Pᵢ to one equilibrating with ATP, it is appropriate to estimate a free energy of interaction. With respect simply to the binding of ligands to a protein, Weber (33) has emphasized that the free energy of interaction is the change in binding free energy for one ligand produced by binding of another ligand and that this free energy change is the same regardless of which ligand is considered first. With respect to (Na⁺, K⁺)-ATPase the free energy of interaction can be considered either as the change in binding free energy for Na⁺ produced by phosphorylation or as the change in the free energy of hydrolysis of the active site phosphate group produced by binding of Na⁺. With the help of several assumptions a semiquantitative estimate of these energies can be made.

The Kₒₜ for Na⁺ with respect to synthesis of ATP from the phosphoenzyme was about 0.6 M (Fig. 3). The Kₒₛ for Na⁺ with respect to inhibition of the dephosphoenzyme from Pᵢ in a steady state is about 0.6 mM (31). Allowing 1.25 kcal per factor of 10 in the concentration ratio at 0° one estimates 3.75 kcal for each Na⁺ bound. Allowing 3 Na⁺ bound per transport cycle (15, 16), the change in free energy of binding of Na⁺ comes out at about 11 kcal.

The Kₒₜ for Na⁺ with respect to phosphorylation in the absence of Na⁺ is about 0.3 M (8, 31). If this value for the insensitive phosphoenzyme is close to that for the sensitive phosphoenzyme, one can estimate the free energy of hydrolysis of the phosphate group at about + (3.5 X 1.25) or + 4.4 kcal. The free energy of hydrolysis of Eᵢ⁻P in the enzyme poisoned with N-ethylmaleimide can be estimated by comparison with that of the terminal phosphate group of ATP, about 7 kcal. Phosphorylation of this form of the enzyme was half-maximal at a ratio of ADP:ATP of 10:31. Thus the free energy of hydrolysis of the phosphate group of Eᵢ⁻P may be about (-7 - 1.25) or -5.75 kcal. The change in free energy of hydrolysis of the phosphate group upon binding of Na⁺ consequently may be about (-5.75 - 4.4) or about -10 kcal (negative sign since ligand is released).

These independent estimates of the free energy of interaction are in reasonable agreement. The agreement supports the idea that interaction free energy provides the energy for synthesis of ATP. The remarkable feature of this enzyme is the extraordinarily high value of the interaction free energy (33).

Considerations from Literature—Fukushima and Tonomura (34) have demonstrated synthesis, or rather resynthesis, of ATP by (Na⁺, K⁺)-ATPase. In their experiment phosphoenzyme, which was formed transiently from ATP, transferred its phosphate group back to ADP upon addition of K⁺. They interpreted their results as indicating that K⁺ favored reversal of transphosphorylation. However, it is known that K⁺ favors dissociation of bound ATP from the enzyme (12, 27, 35) and it is possible that the equilibrium was shifted toward free ATP by this mechanism since dissociation of bound ATP appears to be slow (Fig. 5 and related text).

For energy coupling in oxidative phosphorylation Boyer et al. (36) have offered a new concept. This concept emphasizes that within the confines of an active center transphosphorylation may be an easily reversible reaction and that changes in ligand affinity (33) may be as important as transphosphorylation in the distribution of the total free energy change among the steps of a reaction sequence. Our experience is compatible with this concept.

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