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 μM 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 P_i 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 Na_E_in - Mg.Na.E,kP

K+ ATP+Mg'+ ADP

Na_i Pi + Mg'

IN

Na+ Mg.E2 > Mg.K.E2P

OUT

K+ E2 Mg.K.E2P

OUT

SCHEME 1

Na:E1

K+ ATP+Mg2+

Mg:Na:E1~P

K+:E2

Mg:K+E2:PO

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).

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\textsubscript{i}, respectively. \( E_1 \sim P \) and \( E_2 \sim P \) are the corresponding products of these reactions. The superscript \( s \) indicates that the phosphoenzyme is sensitive to K\textsuperscript{+} and easily attacked by it. In addition to these forms there is also an insensitive phosphoenzyme \( E_3 \sim P \), which appears when the enzyme is incubated with \( P_1 \) and Mg\textsuperscript{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, \( E_1 \sim P \), prepared directly from \( P_1 \) and compare this reaction with that from insensitive phosphoenzyme, \( E_3 \sim P \), 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 Hegvand and Post (12). The amount of enzyme in a sample was estimated by phosphorylation from ATP with Na\textsuperscript{+} and Mg\textsuperscript{2+} under standard conditions (8). The membrane fragments were washed briefly with 1 mm MgCl\textsubscript{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\textsuperscript{+} with ADP and CDTA was added. The reaction was carried out at pH 7.4 and 0\textdegree C unless otherwise stated. The reaction was stopped by addition of 1 ml of 0.37 M trichloroacetic acid containing 74 pmol of HAP\textsubscript{2}O and 1.00 pmol of unlabeled ATP. The denatured membrane suspension was centrifuged at 17,000 \( \times \) g for 20 min at 0\textdegree C and the supernatant was extracted three times with 4 ml of butyl acetate and twice with 4 ml of hexanes at 23\textdegree C. ATP was isolated by column chromatography at 23\textdegree C 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 Tris-HCl (pH 2.5) in the third and fourth chambers of the gradient generator was increased to 150 from 30 mm. The amount of [\textsuperscript{32}P]ATP synthesized was estimated from the ratio of the product of the specific activity of the \( \text{P}_2 \), 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 "differences" refer to the differences between values obtained in the fraction containing the maximum absorbance at 260 nm and those in the second following fraction. The amount of [\textsuperscript{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 [\textsuperscript{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\textsuperscript{+} Concentration on Synthesis of ATP from K\textsuperscript{-}Sensitive or Inensitive Phosphoenzyme**—In earlier experiments from this laboratory (9), the starting material was insensitive phosphoenzyme, \( E_2 \sim P \). 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 \( P_1 \). The experimental basis for the direct preparation of sensitive phosphoenzyme from \( P_1 \) will be presented elsewhere.\textsuperscript{1} The procedure includes the following steps, (a) washing with 1 mm MgCl\textsubscript{2}, (b) removal of free Mg\textsuperscript{2+}, (c) addition of \( P_1 \), and (d) addition of MgCl\textsubscript{2} simultaneously with a low concentration of Na\textsuperscript{+} (4 to 16 mm). The resulting sensitive phosphoenzyme slowly becomes insensitive phosphoenzyme during a period of about 30 s at 0\textdegree C.

A high concentration of Na\textsuperscript{+} was prerequisite for the synthesis of ATP from insensitive phosphoenzyme (9). This requirement was investigated for K\textsuperscript{-}sensitive phosphoenzyme. It was formed and synthesis was started by addition of ADP and CDTA with various concentrations of Na\textsuperscript{+}. After 2 or 80 s the reaction was stopped and phosphoenzyme and ATP were measured. Increase of Na\textsuperscript{+} 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 Na\textsuperscript{+}. After 80 s phosphoenzyme disappeared almost completely, except that in the presence of 15 or 46 mm Na\textsuperscript{+} about 14 or 12% of phosphoenzyme remained, respectively. In the presence of the highest concentrations of Na\textsuperscript{+} 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 Mg\textsuperscript{2+} and 16 mm Na\textsuperscript{+} the results were approximately the same.

To compare the synthesis of ATP from K\textsuperscript{-}sensitive phosphoenzyme with that from insensitive phosphoenzyme in the same enzyme preparation, the enzyme was incubated with \( P_1 \) and 10 mm Mg\textsuperscript{2+} for a longer time to form insensitive phosphoenzyme. In- sensitive phosphoenzyme was less responsive to Na\textsuperscript{+} than K\textsuperscript{-} sensitive phosphoenzyme and the rate of synthesis of ATP was

\textsuperscript{1} G. Toda and R. L. Post, manuscript in preparation.
amol of NaCl was added to start the synthesis of ATP. At 110 a, -0.1 of (Tris)&DTA was added. After the time intervals indicated, the reaction was stopped. The initial amount of phosphoenzyme was estimated from the amount of phosphoenzyme at 2 s at low concentrations of Na+. A calculation was made with the following equation:

$$k_0/k_p = ATP_s/(EP_o - ATP_s)$$

The constant $k_1$ was calculated from the amount of phosphoenzyme at 2 s. In this equation ATPs was the amount of ATP synthesized at 2s and $EP_o$ was the initial amount of phosphoenzyme. At the highest concentrations $k_s$ was considered equal to $k_1$ and $k_p$ was neglected.

From the experimental data from K+-sensitive phosphoenzyme the apparent monomolecular rate constant of ATP synthesis in the presence of various concentrations of Na+ was estimated with an assumption that reactions followed first order kinetics. The rate constant was half-maximal at about 0.6 M and showed saturation at about 1.2 M Na+ (Fig. 3). The corresponding constant for insensitive phosphoenzyme was about 5-fold smaller.

Effect of CDTA on Synthesis of ATP—In most experiments CDTA was used to chelate free Mg* to interrupt phosphorylation from P_i and to stabilise synthesized ATP. To investigate the effect of CDTA; Na+ and ADP with or without CDTA were added to insensitive phosphoenzyme. The reaction was stopped by acid at various times and phosphoenzyme and ATP were measured. The rate of splitting of the phosphoenzyme and both rate and extent of synthesis of ATP were slightly greater in the absence of CDTA (Fig. 4). ATP, once synthesized, was unstable in the absence of CDTA but was very stable in its presence. After disappearance of phosphoenzyme, further in-
creased by 0.60/,, as judged by Dowex 1 column chromatography. The dashed line in Fig. 6 shows the concentration of phosphoenzyme remaining after 40 s was about 30%. This was the synthesis of labeled ATP. In the absence of ADP the amount of ATP synthesized saturated at about 20 μM ADP and was half-maximal at about 6 μM ADP. This result shows that ouabain-insensitive varieties of ATP synthetase were not active in this system.

**Effect of ADP Concentration on Synthesis of ATP**—In order to investigate synthesis of ATP from potassium-complexed phosphoenzyme (Mg-K-E_P) in the absence of CDTA (9). The enzyme was incubated with Pi, 0.125 μmol of MgCl₂, and 1 μmol of KCl to 0.22 ml of a mixture containing 5.0 ml of membrane protein, 5 μmol of imidazole glycylglycine, 0.025 μmol of (Tris) CDTA, and 0.25 μmol of [32P] ATP at pH 7.5 and 0° or 23°. After 6 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 phosphoenzyme at 0 s was taken as 100%. The values in parentheses were obtained at 23°; those outside were obtained at 0°.

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

Lack of ATP synthesis from potassium-complexed phosphoenzyme (Mg-K-E_P)—In order to investigate synthesis of ATP from potassium-complexed phosphoenzyme (Mg-K-E_P), the enzyme was incubated with Pi, Mg₂⁺, and K⁺ at 0° or 23°. ADP and CDTA with or without sodium was added at 6 s and the reaction was stopped with acid at 26 s. The amount of Mg-K-E_P at 6 s at 0° or 23° 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 Mg⁺⁻K⁺-phosphoenzyme (Mg-K-E_P) more rapidly than concentrated Na⁺ converts Mg-E_P into ADP-sensitive phosphoenzyme (Mg-Na-E_P). Another mechanism is that K⁺ reacts directly with Mg-Na-E_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 prevent its conversion into Mg-Na-E_P. Two seconds later, the reaction mixture was diluted 21-fold to reduce the Na⁺ concentration enough to prevent further conversion of Mg-E_P into Mg-Na-E_P, as shown by a preliminary experiment. The diluent 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 P₃, at zero time. At 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 m Na⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>s</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>34</td>
<td>20</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>53</td>
<td>24</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>52</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>36</td>
<td>26</td>
<td>0.3</td>
</tr>
<tr>
<td>12</td>
<td>50</td>
<td>27</td>
<td>1.5</td>
</tr>
</tbody>
</table>

phosphoenzyme remained (Table III). After the dilution, the phosphoenzyme was stable in the absence of K⁺. It was probably mainly Mg·E₂-P since it did not persist in the presence of K⁺. Furthermore, ATP synthesis practically ceased at 3 s following dilution. Nevertheless, the presence of K⁺ did not affect synthesis. In a control sample to which K⁺ was added simultaneously with the 0.68 m Na⁺, no significant synthesis of ATP was observed. The phosphoenzyme which remained after addition of K⁺ with the Na⁺ (57%) was probably mostly K·E₂-P since it was rapidly dephosphorylated after addition of CDTA. In another experiment in which insensitive phosphoenzyme was the starting material, the results were nearly the same as in Table III.

These data and those in Ref. 9 suggest that 16 mM K⁺ attacks Mg·E₂-P more rapidly than 0.68 m Na⁺ converts it into Mg·Na·E₁~P. They suggest further that concentrations of K⁺ below 16 mM do not inhibit the reaction of Mg·Na·E₁~P with ADP.

**Effect of Oligomycin**—Oligomycin is assumed to inhibit the phosphorylation reaction sequence between Mg·Na·E₁~P and Mg·E₂-P (15, 16). In experiments with phosphorylation directly from P₁ oligomycin inhibited Mg·E₂-P formation but not Mg·K·E₂-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 Mg²⁺ 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, 650, and 1920 pg/ml) in the presence of 60 or 160 mM Na⁺. In another experiment the enzyme was incubated with P₁ and Mg²⁺ with or without gramicidin (600 μg/ml) at 35° and pH 7.4 for 20 min and cooled to 0°. At zero time 0.1 ml containing 0.2 μmol of [³²P]ATP was added. At 60 s phosphorylation was started by addition of 0.1 ml containing 0.8 mol of Mg²⁺ without or with 0.8 or 16 mol of NaCl. The reaction was stopped by acid at the times indicated. The amount of phosphoenzyme was estimated (14).

**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.
The effect of phospholipase A treatment was almost complete inhibition of phosphoenzyme formation from ATP or from Pi with or without ouabain and treated or control preparations, respectively. Phosphoenzyme breakdown was also reduced to 60% of the control or 24% of control values, respectively. But the most prominent effect of phospholipase A treatment was almost complete inhibition of ATP synthesis. The rate constant of insensitive phosphoenzyme breakdown was also reduced to 60% of the control (Table V).

<table>
<thead>
<tr>
<th>Source of <strong>P</strong></th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P</strong> <em>pmol/sample</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>176</td>
<td>121</td>
</tr>
<tr>
<td>P1</td>
<td>89</td>
<td>23</td>
</tr>
<tr>
<td>P1 + ouabain</td>
<td>222</td>
<td>54</td>
</tr>
</tbody>
</table>

**ATP synthesis**

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>s</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>9.8</td>
<td>1.2</td>
</tr>
<tr>
<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 P1**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
</table>
| **P1 → ATP Exchange**—Incubation of the enzyme with Na+ and P1 followed by addition of Mg++ completely inhibited phosphoenzyme formation from P1 (8). However, if unlabeled ATP is added with Mg++ in this system, the enzyme accepts phosphate from ATP to form unlabeled phosphoenzyme which exchanges its phosphate group with free Pi (8). In the presence of **P1, radioactive Mg-**E2-P appears. If a high enough concentration of Na+ is present, enough to convert some Mg-E2-P back to Mg-Na-E2-P, **P1**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 **P1 and the phosphorylation reaction was started by addition of Mg++ and

**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 [**P**]. 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 × 100/(100 — EP).

<table>
<thead>
<tr>
<th>Lubrol WX</th>
<th>EP</th>
<th>ATP</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>% (n/y)</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>
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<td>25.0</td>
</tr>
<tr>
<td>1.92</td>
<td>31.0</td>
<td>14.7</td>
<td>21.3</td>
</tr>
</tbody>
</table>

**Table V**

*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 X g for 45 min). The samples were suspended in a solution containing 10 mM imidazole, 2 mM HCl, and 0.1 mM H,EDTA and packed as before. The precipitates were suspended in 1 ml of 10 mM imidazole-HCl buffer containing 0.1 mM H,EDTA (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 0.043 _μmol_ of [**P**]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 0.15 ml of control or treated preparation, 5 _μmol_ of imidazole glycylglycine, and 0.5 _μmol_ of **P** 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 160 _μmol_ of NaCl, 0.5 _μmol_ of ADP, 0.005 _μmol_ of ATP, and 10 _μmol_ of (Tris)2CDTA to the sample phosphorylated from P1. After the time intervals indicated, 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.
The amount of ATP recovered was or without ouabain, and only moderately decreased phosphorylation from ATP. The reaction was started by acid at 50 s after starting the reaction. [**P]ATP and [**P]phosphoenzyme were estimated. When present, 0.25 μmol of ouabain was added with Mg** and ATP. In some samples, 160 mM NaCl was replaced by 16 mM NaCl or 160 mM KCl. The amount of phosphoenzyme obtained 30 s after starting the reaction in the complete system was taken as 100%.

**Table VI**

Incorporation of **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 μmol of **P Pi, 160 μmol of NaCl, and 10 μmol of imidazole glycylglycine in a final volume of 0.8 ml overnight at pH 7.5 and 0°C. The reaction was started by addition of 0.1 ml containing 2 μmol of MgCl2 and 0.01 μmol of unlabeled ATP. After 30 s 0.1 ml containing 1 μmol of ADP with 20 μmol of (Tris)CDTA was added. The reaction was stopped by acid at 50 s after starting the reaction. [**P]ATP and [**P]phosphoenzyme were estimated. When present, 0.25 μmol of ouabain was added with Mg** and ATP. In some samples, 160 mM NaCl was replaced by 16 mM NaCl or 160 mM KCl. The amount of phosphoenzyme obtained 30 s after starting the reaction in the complete system was taken as 100%.

Incorporation of **P from Pi into ATP in presence of ATP and high concentration of Na**

<table>
<thead>
<tr>
<th>Incubation with</th>
<th>[**P]Phosphoenzyme</th>
<th>[**P]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>1.0</td>
<td>0</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Reversal of (Na** K**)-ATPase Reaction via Functional Phosphoenzyme—These experiments showed net synthesis of ATP from ADP and Pi. The amount of [**P]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** 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** (Scheme 1). Reversibility of this step in a steady state was indicated by a slow ouabain-inhibitable P** 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 into E** P (15, 16). Synthesis of ATP did not appear to require free Mg** as if the Mg** required for the initial phosphorylation remained bound to the phosphoenzyme (8). Synthesis was remarkably sensitive to ADP with a K** about 12 μM. This value is higher than the dissociation constant from the dephosphoenzyme in the presence of Na**, 0.34 μM (27) or in the absence of Na**, 2 μM (12) or 1 μM (28).
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 contrast 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 reversible in = out exchange of Na⁺ through the pump in human erythrocytes, Garay and Garrahan (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 K₀.₅ by multiplying them by the reciprocal of (2¹/₂ − 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 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 heterogeneous 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 almost equally effective whether it was added to the reaction mixture early or late (Fig. 8). (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 Mg⁺⁺ (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 K₀.₅ for Na⁺ with respect to synthesis of ATP from the phosphoenzyme was about 0.6 M (Fig. 3). The K₀.₅ 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 K₀.₅ for Pᵢ with respect to phosphorylation in the absence of Na⁺ is about 0.3 mM (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 × 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 re-synthesis, 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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REFERENCES

Synthesis of adenosine triphosphate and exchange between inorganic phosphate and adenosine triphosphate in sodium and potassium ion transport adenosine triphosphatase.

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