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Kinetic Properties of Phosphorylated Intermediates in the Reaction of \( \text{Na}^{+}-\text{K}^{+} \)-dependent ATPase

Yoshihiro Fukushima

Department of Biology, Faculty of Science, Osaka University
The transport of Na\(^+\) out of the cell and K\(^+\) into the cell is a fundamental energy-requiring cellular process to perform physiological functions such as absorption in kidney and intestine, excitability in nerve and muscle, and the maintenance of cell volume (1,2). The hydrolysis of ATP provides the energy for the transport of cations (1,2). Shou (3) first discovered in the microsomal fraction of nerve a Mg\(^{2+}\)-requiring adenosinetriphosphatase which was markedly activated by simultaneous presence of Na\(^+\) and K\(^+\).

Since then numerous lines of evidence (4-9) have supported that this membrane-bound enzyme system, Na\(^+\)-K\(^+\)-dependent ATPase [EC 3.6.1.3.], is involved in the active transport of Na\(^+\) and K\(^+\) in opposite directions across the plasma membrane. Using \(\gamma^{32}\)P-labeled ATP, several workers (10-19) showed that Na\(^+\)-K\(^+\)-dependent ATPase is phosphorylated in the presence of Mg\(^{2+}\) and Na\(^+\) by the transfer of the \(\gamma\)-terminal phosphate group of ATP to the enzyme, and the phosphorylated enzyme is hydrolyzed when K\(^+\) is added. Much evidence (2,10-23) has been accumulated to show that the phosphorylated enzyme is a reaction intermediate of Na\(^+\)-K\(^+\)-dependent ATPase from the steady-state kinetics.

However, several important points remain to be clarified to understand in molecular terms how Na\(^+\)-K\(^+\)-dependent ATPase works for the active transport of cations. Fahn, Albers, and their colleagues (20,21) proposed the following reaction mechanism, based on studies on the ATP-ADP exchange reaction
catalyzed by the ATPase preparation:

\[ E + ATP \rightleftharpoons E\cdot ATP \rightleftharpoons E_1\cdot P + ADP \rightarrow E_2\cdot P + ADP \rightarrow E + P_i + ADP, \]

where \( E_1\cdot P \) is a high energy phosphorylated intermediate which can react with added ADP to form ATP, and \( E_2\cdot P \) is a low energy phosphorylated intermediate which does not react with added ADP and which is hydrolyzed in the presence of \( K^+ \). They considered that almost all the phosphorylated intermediate is in the form of \( E_2\cdot P \) in the presence of high concentrations of \( Mg^{2+} \), while both \( E_1\cdot P \) and \( E_2\cdot P \) coexist in the presence of low concentrations of \( Mg^{2+} \), especially when NEM-treated enzyme is used. This reaction mechanism was supported by the subsequent kinetic studies of Post et al. (22). Post et al. also suggested that phosphate is bound to the same site on the enzyme in the two phosphorylated intermediates, and considered that \( E_1\cdot P \) undergoes an irreversible, \( Mg^{2+} \)-dependent conformational transition to \( E_2\cdot P \). We will refer to this reaction mechanism as the F.A.P. mechanism.

On the other hand, Kanazawa, Saito, and Tonomura (24, 25) investigated the partial reactions, i.e., the formation and decomposition of the phosphorylated intermediate, and compared their kinetic properties with those of the overall reaction in the presence of a high concentration of \( Mg^{2+} \) (i.e. 1 mM). From the results, they proposed the following reaction mechanism:

\[ E + ATP \rightleftharpoons E_1\cdot ATP \rightarrow E_2\cdot ATP \rightleftharpoons E\cdot ADP \rightarrow E + P_i + ADP, \]

step 1 \hspace{1cm} 2 \hspace{1cm} 3 \hspace{1cm} 4
where the phosphorylated intermediate is a high energy form, and is in K⁺-dependent equilibrium with E₂·ATP. From the results obtained by Post et al. (22), it appears that only an ADP-insensitive phosphorylated intermediate exists in the presence of 1 mM Mg²⁺. Therefore, the phosphorylated intermediate in the above mechanism is a high energy type with bound ADP, Eₚ⁻ADP, although the state of ADP is not specified in the paper of Kanazawa et al. (24). Thus the following mechanism may be proposed for Na⁺-K⁺-dependent ATPase reaction under conditions where both the ADP-sensitive and the ADP-insensitive phosphorylated intermediates are formed:

\[
E + ATP \rightleftharpoons E₁·ATP \rightleftharpoons E₂·ATP \rightleftharpoons Eₚ⁻ADP \rightleftharpoons E·P + ADP \Rightarrow E + Pi + ADP.
\]

We will refer to this reaction mechanism as the modified K.S.T. mechanism.

Criteria to discriminate between the above two mechanisms can be derived from the following two experiments. One is the addition of K⁺ to the EP* formed in the presence of high concentrations of Mg²⁺. Based on the F.A.P. mechanism this will only promote Pi-liberation from EP. However, according to the K.S.T. mechanism, rapid ATP-formation will be observed due to a shift of the equilibrium of step 3 toward the

* The abbreviations used are: EP, phosphorylated intermediate; TCA, trichloroacetic acid; NEM, N-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; AT³²P, P³²P-labeled ATP.
formation of $E_2\cdot$ATP, with rapid decay of EP immediately after
the addition of $K^+$, in addition to the promotion of $P_i$-libera-
tion by EP-decomposition. The second experiment is the
addition of ADP to the EP of NEN-treated enzyme in the presence
of low concentrations of $\text{Mg}^{2+}$, resulting in the rapid decay
of a fraction of the EP. According to the F.A.P. mechanism,
the ADP-sensitive fraction of EP will decrease with time
during the reaction, while according to the modified K.S.T.
mechanism it will increase with time to reach a steady-state
level. The first section of the "RESULTS AND DISCUSSION"
describes the results of these two types of experiments,
which strongly support the modified K.S.T. mechanism.

It is important to know the effects of Na$^+$ and K$^+$ on
the elementary steps of Na$^+$-K$^+$-dependent ATPase to understand
the molecular mechanism of active transport of cations.
Although the overall kinetics of Na$^+$- and K$^+$- activation of
the enzyme reaction have been examined by many investigators
(1,2,23,26,33), kinetic analysis of the effects of cations
on the elementary steps has not been studied systematically
because of the difficulty of following the rapid reaction.
Therefore, we analyzed the effects of Na$^+$ and K$^+$ on
the elementary steps of the ATPase reaction according to the
modified K.S.T. mechanism in the wide ranges of Na$^+$ and K$^+
concentrations. Thus, the effects of Na$^+$ and K$^+$ on the
elementary steps are described in the second section of the
"RESULTS AND DISCUSSION".
The existence of the equilibrium between $E_2\cdot$ATP and $E_2\cdot$ADP (step 3) is one of the characteristic features in the K.S.T. mechanism. The coupling of the translocation of cations across the membrane with the step from the enzyme-ATP complex to the phosphorylated intermediate was demonstrated (25,34,35) on vesicular sarcoplasmic membrane preparation. Furthermore, the existence of a similar equilibrium step was also shown (36,37) in the reaction of myosin-ATPase. In the case of myosin-ATPase, the second form of myosin-ATP complex, $E_2\cdot$ATP, is in an equilibrium with the reactive myosin-phosphate-ADP complex, $E_2\cdot$ADP. The thermodynamic functions of this equilibrium step were recently obtained (37). In view of the general mechanism of the biological energy transducing processes, it is important to know the properties and the thermodynamic functions of the step $E_2\cdot$ATP $\rightleftharpoons E_2\cdot$ADP in the reaction of Na$^+$-K$^+$-dependent ATPase. Therefore the thermodynamic parameters of the step 3 in the K.S.T. mechanism are given in the third section of the "RESULTS AND DISCUSSION" and they are compared with those of the myosin-ATPase reaction.

EXPERIMENTAL PROCEDURE

Preparation of Na$^+$-K$^+$-dependent ATPase — Gray matter from bovin brain was homogenized with 10 volumes of 0.32 M sucrose containing 1 mM EDTA at pH 7.4 and then centrifuged at 10,000 x g for 15 min. The supernatant was then centrifuged
at 20,000 x g for 60 min. The resulting pellet was resuspended in about 10 volumes of the above sucrose solution and centrifuged again at 10,000 x g for 15 min. The supernatant was treated with a high concentration of NaI solution following the method of Nakao et al. (38). The enzyme thus obtained was washed 4 times with 0.1 M sucrose containing 1 mM EDTA at pH 7.4 to remove NaI, and then once with 0.1 M sucrose containing 1 mM Tris-HCl at pH 7.4 to remove traces of EDTA. All these manipulations were performed at 0°C. The washed enzyme was stored at -20°C in 0.1 M sucrose containing 1 mM Tris-HCl at pH 7.4. The ATPase activity was stable for more than 6 months in frozen suspensions. Aliquots were slowly thawed and diluted with buffer just before use. After thawing, the enzyme was stable at 0°C for several hours in 0.1 M sucrose containing 1 mM Tris-HCl at pH 7.4. The concentration of protein was determined by the method of Lowry et al. (39) with bovine serum albumin as standard.

Preparation of Chemicals — Sodium salt of ATP and ADP were purchased from Kyowa Hakko Co. and converted to Tris-ATP by passage through an Amberlite IR-120 column at pH 7.4 with Tris-HCl as solvent. ATP was prepared by the method of Glynn and Chappel (40), and used as the Tris-salt. The specific radioactivity of the preparation obtained was $10^8$ to $10^9$ cpm/μ mole of ATP. NEM was purchased from Sigma Chemical Co. It was dissolved in ethanol at a concentration of 0.1 M just before use, and diluted with distilled water
Charcoal was purchased from Takeda Chemical Co. It was boiled successively for 30 min in 50 and 100 % acetic acid, washed with distilled water, and boiled again in distilled water for 45 min. The washing and boiling in distilled water were repeated 3 times, and then the charcoal was dried in vacuo. It was heated in a crucible for 2-3 hrs before use. Other reagents were of reagent grade.

**Phosphorylation Reaction**—The phosphorylation reaction was started by the addition of ATP$^{32}$P to the reaction mixture containing the enzyme and other reagents under the conditions given in the "RESULTS AND DISCUSSION". At different times after starting the reaction, it was stopped by addition of TCA (final concentration of 5 %) containing P$_i$ (final concentration of 40 $\mu$M) and carrier ATP (final concentration of 0.4 $\mu$M). The reaction was followed using the simple mixing apparatus, which is driven by solenoids, devised by Kanazawa *et al.* (24). Three ml of ice-cold 4 % perchloric acid containing 1 mM carrier ATP were added to the mixture immediately after stopping the reaction, and the amount of phosphorylated protein was determined as described below.

**Dephosphorylation Reaction**—After starting the phosphorylation reaction, it was terminated by addition of EDTA (Tris-salt, pH 8.5) or excess unlabeled ATP. At different times after the addition of EDTA or carrier ATP, the dephosphorylation reaction was stopped by the addition of TCA containing P$_i$ and carrier ATP. Subsequent procedures were similar to those
used for the phosphorylation reaction. The dephosphorylation reaction was also followed using the simple mixing apparatus. **Determination of Phosphorylated Protein**—After stopping the reaction, the suspension of denatured enzyme was charged on a Millipore filter of 0.8 μm pore size, and washed twice with 5 ml of 4% perchloric acid containing 20 mM Pi and 1 mM carrier ATP, and then 10 times with 5 ml of 4% perchloric acid containing 20 mM Pi and finally twice with 5 ml of water. All the procedures were performed at 5°C. The washed precipitate on the filter was heated at 100°C for 2 min in 4 ml of 0.01 N NaOH containing 0.1 mM Pi. In this way essentially all the phosphorylated protein was decomposed liberating 32Pi. An aliquot of the extract obtained by the Martin-Doty method (41) was analyzed for radioactivity. **Determination of Pi**—After stopping the reaction, the suspension of denatured enzyme was centrifuged, and 32Pi in the supernatant was extracted by the method of Martin and Doty (41), and its radioactivity was measured. **Determination of ATP**—After stopping the reaction, the suspension of denatured enzyme was centrifuged, and 32Pi in the supernatant was extracted by the method of Martin and Doty (41). The upper phase of isobutanol-benzene was discarded to remove Pi. This extraction procedure was repeated 4 times. The resulting lower phase was stirred vigorously with about 10 mg of charcoal in a flush mixer for 5 min, and then filtered through a Millipore filter (pore size
0.45 μ). The charcoal on the filter was again stirred with 2 ml of NH₄OH-ethanol solution (1%: 50%) for 2 min in a flush mixer to extract adsorbed nucleotides, and then the mixture was filtered through a Millipore filter to remove the charcoal. The radioactivity of a portion of the filtrate was measured. Preliminary tests showed that under these experimental conditions all the radioactivity measured by the above method was due to AT³²P, and that the overall recovery of AT³²P was about 86%. This value for recovery was used in making corrections to obtain the true amount of AT³²P in samples.

**Treatment of Enzyme with NEM**—An enzyme preparation (0.68 mg/ml protein) was exposed to 1 mM NEM in 0.1 M Tris-HCl, pH 8.4, at 15°C for 1 hr. Treatment with NEM inhibited Na⁺-K⁺-dependent ATPase activity by 80-95%, but did not inhibit EP-formation.

**Analysis of Kinetic Data**—The kinetic data were analyzed following Kanazawa et al. (24). The equations employed in the present study are outlined below. The first order decay constant of EP-disappearance, kₐ, can be estimated from the exponential disappearance of E³²P with time after addition of excess EDTA or unlabeled ATP to stop E³²P-formation. The rate constant of EP-decomposition at step 4, k₊₄, is given as the ratio of the rate of ATP-hydrolysis, Vₒ, to the EP concentration, [EP], in the steady-state,

\[ k₊₄ = \frac{Vₒ}{[EP]} \] (1).
The rate of EP-disappearance after stopping its formation is given by the equation

\[ -\frac{d[EP]}{dt} = k_d[EP] = \frac{k_{+4}[EP]}{1 + K_3} \]  

(2),

where \( K_3 \) is the reciprocal of the equilibrium constant of step 3, i.e. the ratio of the concentration of \( E_2 \cdot ATP \) to that of EP. Hence, \( K_3 \) is given by

\[ K_3 = \frac{V_0}{[EP]} - 1 \]  

(3).

If we assume that steps 1 and 3 reach equilibrium rapidly compared to step 2, the rate of \( E_2 \cdot ATP \)-formation, \( V_f \), can be calculated from the observed rate of EP-formation, \( V_f' \), as:

\[ V_f = V_f'(1 + K_3) \]  

(4).

RESULTS AND DISCUSSION

Reaction Mechanism of

\( Na^+ - K^+ \)-dependent ATPase (42,43)

Evidence for a High Energy Phosphorylated Intermediate with Bound ADP in the Presence of High Mg\(^{2+}\) Concentration

To demonstrate that not all the EP formed in the reaction of the \( Na^+ - K^+ \)-dependent ATPase preparation reacted with added ADP in the presence of high concentrations of Mg\(^{2+}\), the phosphorylation reaction was started with 10 \( \mu \)M \( AT^{32}P \) in the presence of 5 mM MgCl\(_2\), 100 mM NaCl and 0.14 mg/ml of
enzyme at pH 7.5 and 0°C. Then 3.1 mM ADP and 2.7 mM MgCl₂ or 50 mM EDTA was added to the reaction mixture 0.2 or 2 sec after starting the phosphorylation reaction. As shown in Fig. 1, the time-course of EP-decay after adding Mg-ADP was identical with that after adding EDTA both 0.2 and 2 sec after starting the reaction.

According to the K.S.T. mechanism, there is a K⁺-dependent equilibrium between E₂·ATP and EP, and it rapidly shifts toward formation of E₂·ATP on adding K⁺. To see if this was so, ATP-formation after addition of K⁺(K⁺-jump) to EP was examined by DEAE-Sephadex column chromatography. The enzyme (3.5 mg/ml) was phosphorylated in the presence of 5 mM MgCl₂ and 100 mM NaCl at pH 8.5 and 15°C using 0.1 μM ATP³²P to keep the level of free ATP as low as possible. Under these conditions, the amount of EP reached a maximum 2 sec after starting the reaction, and then gradually decreased. Two sec after starting the reaction, 0.77 mM KCl containing 50 mM EDTA was added, and 0.5 sec later TCA was added. In the control experiment, TCA was added 2 sec after starting the reaction. Pᵢ was removed from the two samples, using the method of Martin and Doty (41). Nucleotides were absorbed on charcoal, extracted with NH₄OH-ethanol solution,
and then charged on a column, as described in the "EXPERIMENTAL PROCEDURE". Figure 2 shows that the amount of ATP increased after adding K⁺. Thus, the total amount of ATP after addition of K⁺ was about 1.7 times that of the control*. All the radioactivity appeared in the same position as the absorption of carrier ATP**. Therefore, it was not necessary to separate ATP by column chromatography and we could follow the time-course of ATP-formation after addition of K⁺ by direct measurement of the radioactivity of the sample extracted from charcoal.

The phosphorylation reaction was performed as for Fig. 2, except that 150 mM NaCl, 1.63 mg/ml enzyme and 0.05 μM ATP³² were used. As shown in Fig. 3, the amount of ATP formed increased with time, and reached a maximum of 1.2 moles/10⁹ g protein, 0.6 sec after adding 0.77 mM KCl and 50 mM EDTA.

* The background count was taken as 16 cpm.
** This nucleotide preparation was usually contaminated with a small amount of some substance, with absorption at 260 nm.
On the other hand, the amount of EP first decreased rapidly after adding KCl and EDTA, and then decreased, following first-order kinetics. (The amount of EP is plotted as the ordinate on a logarithmic scale in Fig. 3.) The amount of EP also decreased, following first-order kinetics after adding 50 mM EDTA, and the rate constant of the decay in the absence of K⁺ was about 70% of that in the presence of 0.77 mM K⁺.

The reciprocal of the equilibrium constant of step 3 in the K.S.T. mechanism in the presence of 0.77 mM K⁺ was calculated to be 0.83-0.93, both from the amount of rapid decrease of EP after adding KCl and EDTA and from the amount of ATP formed, when the reciprocal of the equilibrium constant in the absence of K⁺ was taken as 0.15 according to Kanazawa et al. (24). This value for the constant in the presence of 0.77 mM KCl was almost equal to the value of about 1.0 of Kanazawa et al. (24) calculated indirectly from the rate constant of Pᵢ-liberation and the first-order rate constant of EP-decay in the presence of 0.3 mM KCl.

These results clearly show that EP formed in the presence of high concentrations of Mg²⁺ is a high energy type with bound ADP, i.e. E₋^ADP. Evidence for Sequential Formation of Two High Energy Phosphorylated Intermediates With and Without Bound ADP

In studies on the order of formation of ADP-sensitive and ADP-insensitive EP, we used the conditions where both types
of EP coexist in the steady-state. It has been reported by several workers (20-22,44) that two types of EP coexist in the presence of low concentration of \( \text{Mg}^{2+} \). Figure 4 shows the time-course of \( \text{E}^{32}\text{P} \)-decay after adding 0.45 mM \( \text{Mg-ADP} \), and that after adding 0.45 mM unlabeled \( \text{Mg-ATP} \), in the presence of 5 \( \mu \text{M} \) \( \text{AT}^{32}\text{P} \), 0.156 mg/ml enzyme and 140 mM NaCl at pH 7.5 and 15°C and in the presence of a low concentration of \( \text{Mg}^{2+} \) of 10 \( \mu \text{M} \). We could observe no acceleration of EP-decay on addition of ADP even in the presence of 10 \( \mu \text{M} \) \( \text{Mg}^{2+} \).

Fahn et al. (21) reported that NEM-treatment accelerates the ATP-ADP exchange reaction catalyzed by the enzyme. Accordingly a similar experiment was performed using NEM-treated enzyme. NEM-treated enzyme was prepared as described in the "EXPERIMENTAL PROCEDURE", and phosphorylated by incubation for 1 sec with 10 \( \mu \text{M} \) \( \text{AT}^{32}\text{P} \) in the presence of 10 \( \mu \text{M} \) \( \text{MgCl}_2 \) and 100 mM NaCl at pH 7.5 and 0°C. As shown in Fig. 5,

the rate of EP-decay of the NEM-treated enzyme after adding 1 mM \( \text{Mg-ADP} \) was considerably faster than that after adding
50 mM EDTA. The rates of EP-decay after adding EDTA and ADP under these conditions were rather low, and the time-courses of EP-decay in the steady-state were nearly linear with time. Hence, the difference between the linear parts of the curves obtained on adding EDTA and ADP, respectively, could be regarded as the amount of ADP-sensitive EP formed at the time when ADP was added. The amount of ADP-sensitive EP was estimated as about 56 % of the total EP, 1 sec after starting the reaction under the conditions used in the experiments shown in Fig. 5.

The order of formation of the two kinds of EP was followed. The phosphorylation reaction was performed under the same conditions as for Fig. 5. At various times after starting the phosphorylation reaction, 50 mM EDTA or 3.1 mM ADP containing 2.7 mM MgCl₂ was added, and the time-course of EP-decay was followed. It is clear from Fig. 6 that the acceleration of EP-decay on addition of ADP occurs after

Figure 6

0.4 sec reaction, not in the initial phase of the phosphorylation reaction (0.1 and 0.2 sec). The ratio of the amount of ADP-sensitive EP to the total amount of EP was estimated as described above. As shown in Fig. 7, the percentage amount of ADP-sensitive EP was very small in the initial
phase of the reaction, and increased with time. It reached a constant level of about 40 %, 1.5 sec after starting the reaction. These results clearly show that first the ADP-insensitive EP, and then the ADP-sensitive EP is formed, at least under conditions where both types of EP can exist.

The results of the two experiments described above strongly support the modified K.S.T. mechanism, but not the F.A.P. mechanism. According to the former mechanism step 2 is essentially irreversible and step 3' is rate-limiting in the presence of high concentrations of Mg$^{2+}$ (24). On the other hand, when NEM-treated enzyme is used in the presence of low concentrations of Mg$^{2+}$, step 2 is reversible, the rates of steps 3 and 3' are similar, and both $E<^{ADP}$ and $E\sim P$ can coexist in the steady-state. Formation of EP is known to require both Na$^+$ and Mg$^{2+}$. In addition, it must be noted that there is a K$^+$-dependent equilibrium between the enzyme-ATP complex and the phosphorylated intermediate, as first indicated by Kanazawa et al. (24,25) and confirmed in this study. Therefore, EP-formation is affected not only by Na$^+$ but also by K$^+$, as recently reported by Siegel et al. (45) and Banergi et al. (46).
Effects of Sodium and Potassium Ions on the Elementary Steps in the Reaction of Na\(^+\)-K\(^+\)-dependent ATPase (43,47)

In this section, the effects of Na\(^+\) and K\(^+\) on the partial reactions of Na\(^+\)-K\(^+\)-dependent ATPase in the presence of high concentrations of Mg\(^{2+}\) are described, since the reaction mechanism at high Mg\(^{2+}\) concentrations is simpler than that at low Mg\(^{2+}\) concentrations as described in the first section. As reported by Kanazawa et al. (24,25), step 1, is not affected by Na\(^+\). Therefore, the effects of Na\(^+\) and K\(^+\) on the other step, i.e. step 2, 3, and 4, were investigated employing equations (4), (3), and (1), respectively.

**Effects of Na\(^+\) and K\(^+\) on EP-decomposition** — About 1 sec after initiating the reaction by adding ATP to the enzyme, the ATPase reaction reached the steady-state. The amounts of P\(_i\) liberated and EP formed in the 3 sec - reaction were measured to obtain the rate constant of EP-decomposition at step 4 as \(v_0/[EP]\) (cf. eq. (1)).

Figure 8 shows the dependence of the value of \(v_0/[EP]\) on the concentration of NaCl in the absence of KCl. The reaction was performed using 0.29 mg/ml enzyme and 4 \(\mu\)M ATP. The rate of ATP-hydrolysis, \(v_0\), was about 0.55 mole P\(_i\)/10\(^7\) g
protein/sec with 0.5 mM NaCl, and increased with increase in the concentration of NaCl. It reached a maximum value of about 1.8 mole P_i/10^7 g protein/sec with 10-20 mM NaCl, and then decreased with increase in the concentration of NaCl to a value of about 0.4 mole P_i/10^7 g protein/sec with 400 mM NaCl. The concentration of EP, [EP], was about 0.5 mole/10^7 g protein with 0.5 mM NaCl, and increased with increase in the concentration of NaCl. It reached about 0.95 mole/10^7 g protein with 5 mM NaCl and maintained a constant level of 0.95-1.1 mole/10^7 g protein in the range of 5 to 400 mM NaCl. Accordingly, the rate constant of EP-decomposition at step 4, calculated as V_o/[EP], showed approximately a constant value of 1.3 sec^{-1} in 0.5-50 mM NaCl, and decreased with further increase in the NaCl concentration to a value of about 0.4 sec^{-1} with 400 mM NaCl.

The dependences of V_o/[EP] on the concentration of NaCl were measured in the presence of 0.1, 0.5, 2.5, and 10 mM KCl. Figure 9 shows the results obtained in the presence of 2.5 mM KCl. The reaction was performed using 0.29 mg/ml enzyme and 40 µM ATP. As expected, the rate of ATP-hydrolysis in the presence of 2.5 mM KCl was larger than that in the absence of KCl (cf. Fig. 8). As shown in Fig. 9, the value of V_o was
about 2.5 moles $P_i/10^7$ g protein/sec with 1 mM NaCl, and increased with increase in the concentration of NaCl. It reached a maximum value of about 11.5 moles $P_i/10^7$ g protein/sec with 50 mM NaCl, and then decreased with increase in the concentration of NaCl to a value of about 0.5 mole $P_i/10^7$ g protein/sec with 600 mM NaCl. The dependence of [EP] on NaCl concentration in the presence of 2.5 mM KCl gave a biphasic curve. The value of [EP] was less than that in the absence of KCl, especially at low concentrations of NaCl (cf. Fig. 8). It was about 0.15 mole/10^7 g protein with 1 mM NaCl, and increased with increase in the concentration of NaCl. It showed a plateau of about 0.6 mole/10^7 g protein in 50-100 mM NaCl, increased again with further increase in the NaCl concentration, and reached about 0.95 mole/10^7 g protein with 600 mM NaCl. Accordingly, the rate constant of EP-decomposition, $k_{o}/[E_P]$, showed an approximately constant value of 20 sec$^{-1}$ in 1-10 mM NaCl, and decreased with further increase in the concentration of NaCl. With 600 mM NaCl it was only about 2 sec$^{-1}$. The biphasic dependence of [EP] on the concentration of NaCl was more marked in the presence of 10 mM KCl than that shown in Fig. 9 in the presence of 2.5 mM KCl.

The dependences of the rate constant of EP-decomposition on NaCl concentration at various KCl concentrations are given in Fig. 10. At low concentrations of NaCl, EP-decomposition was activated markedly even by addition of a low
concentration of KCl. The inhibition of EP-decomposition by high concentrations of NaCl was partially suppressed by KCl. The curves illustrated in Fig. 10 were calculated by the following equation:

\[
\frac{V_0}{[EP]} = \frac{V_{0,K}}{[EP]} + \frac{18.4}{(1 + \frac{0.043 \text{ mM}}{[K^+]})} (\text{sec}^{-1})
\]

\[
1 + \frac{[Na^+]}{130 [K^+]} + \left(\frac{[Na^+]}{190 \text{ mM}}\right)^2
\]

where \(\frac{V_0}{[EP]}\)\(_{K=0}^+\) denotes the value of \(\frac{V_0}{[EP]}\) in the absence of KCl. As seen in Fig. 10, all the experimental values fitted the above equation well.

The dependence of the value of \(\frac{V_0}{[EP]}\) at a fixed concentration of NaCl on the concentration of KCl was also measured. Figure 11 shows the dependence in the presence of 10 mM NaCl. The values of \(\frac{V_0}{[EP]}\) with various concentrations of KCl in 10 mM NaCl were similar to those given in Fig. 10.
Effects of Na\(^+\) and K\(^+\) on the Equilibrium Step between E\(_2\)ATP and EP. Employing the first order rate constant of EP-disappearance, K\(_d\), and V\(_o\)/[EP], we calculated the value of 1 + K\(_3\) as V\(_o\)/([EP] k\(_d\)), and measured the effects of Na\(^+\) and K\(^+\) on K\(_3\), which is the ratio of the concentration of E\(_2\)ATP to that of EP (cf. eq. (3)).

Figure 12 shows the dependence of the value of 1 + K\(_3\) on the concentration of NaCl in the absence of KCl. To measure the value of k\(_d\), the ATPase reaction was performed with 0.29 mg/ml enzyme and 4 \(\mu\)M ATP in the presence of 0.5-400 mM NaCl. Three sec after initiating the reaction, 50 mM EDTA was added to the reaction mixture to stop EP-formation. The value of k\(_d\) was estimated from the slope of the plot of the logarithm of the EP-concentration after addition of EDTA against time. The value of V\(_o\)/[EP] was obtained as described for Fig. 8. The value of k\(_d\) was nearly independent of the concentration of NaCl at concentrations of 0.5-400 mM, being about 0.5 sec\(^{-1}\), although in 50-80 mM NaCl it had a slightly larger value of 0.65 sec\(^{-1}\). On the other hand, the value of V\(_o\)/[EP] was about 1.3 sec\(^{-1}\) in 0.5-50 mM NaCl, and decreased with increase in the NaCl concentration to 0.5 sec\(^{-1}\) with 400 mM NaCl. Accordingly, the value of
$1 + K_3$ calculated as $V_0/([EP] \cdot k_d)$ showed an approximately constant value of 2.8-2.9 in 0.5-10 mM NaCl, and decreased with increase in the concentration of NaCl to about 1 in 100-400 mM NaCl.

Figure 13 shows the dependence of the value of $1 + K_3$ on the concentration of NaCl in the presence of 2.5 mM KCl.

The reaction was performed using 0.29 mg/ml enzyme and 40 μM ATP. As seen in this figure, the value of $V_0/[EP]$ was approximately constant at 18-20 sec$^{-1}$ in 1-80 mM NaCl, and decreased with further increase in the concentration of NaCl to about 1 sec$^{-1}$ in 400-600 mM NaCl. The value of $k_d$ was about 5 sec$^{-1}$ in 1-10 mM NaCl, and decreased with increase in the concentration of NaCl to about 1 sec$^{-1}$ with 400 mM NaCl. Accordingly, the value of $1 + K_3$ was about 4 with 1 mM NaCl, reached a maximum value of about 13 with 90 mM NaCl, and then decreased to 1 with further increase in the concentration of NaCl to 400-600 mM NaCl.

Figure 14 shows the dependence of the value of $1 + K_3$ on the concentration of NaCl in the presence of 10 mM KCl.
The reaction was performed under the same conditions as for Fig. 13, except that the concentration of KCl was 10 mM. The value of \( \frac{v_0}{[E]P} \) was approximately constant at 18 sec\(^{-1} \) in 1-50 mM NaCl, and decreased with increase in the concentration of NaCl to about 1 sec\(^{-1} \) with 600 mM NaCl. The value of \( k_d \) was approximately constant at 1-1.3 sec\(^{-1} \) in 1-600 mM NaCl. Accordingly, the value of \( 1 + K_3 \) was 12 in 1-50 mM NaCl, and decreased with further increase in the concentration of NaCl to about 1 with 600 mM NaCl.

Figure 15 shows the dependences of the value of \( 1 + K_3 \) on the concentration of NaCl in the presence of 0, 0.5, 2.5 and 10 mM KCl. Although the dependence in the presence of 2.5 mM KCl was complicated, as mentioned above (cf. Fig. 13), generally speaking, the value of \( 1 + K_3 \) decreased with increase in the concentration of NaCl, and increased with increase in the concentration of KCl.

As shown in Fig. 11, the dependence of the value of \( 1 + K_3 \) on the concentration of KCl in the presence of 10 mM NaCl approximately agreed with the results shown in Fig. 15. Effects of Na\(^+\) and K\(^+\) on E\(_2\)ATP-formation — The rate of E\(_2\)ATP-formation, \( \psi_f \), was calculated as \( \psi_f \) \( (1 + K_3) \) (cf. eq. (4)), where the rate of EP-formation, \( \psi_f' \), was obtained from the
amount of EP formed in 0.1 sec after initiating the reaction.

Figure 16 shows the dependence of the value of $v_f$ on the concentration of NaCl in the absence of KCl. The reaction was performed using 0.29 mg/ml enzyme and 40 μM ATP. The value of $1 + K_3$ shown in Fig. 15 was used to calculate the value of $v_f$. The value of $v_f$ was about 2 moles/10^7 g protein/sec with 1 mM NaCl, and increased with increase in the concentration of NaCl. It reached a maximum value of 20 moles/10^7 g protein/sec with 20 mM NaCl, and then decreased with further increase in the concentration of NaCl to about 2 moles/10^7 g protein/sec with 600 mM NaCl. The solid line in Fig. 16 was calculated by the equation:

$$v_f = \frac{43}{104 \text{ mM} + \frac{[\text{Na}^+]}{31.2 \text{ mM}}} \text{ (moles/10}^7 \text{ g protein/sec)} \quad (6).$$

The observed values of $v_f$ fitted the curve approximately.

Figure 17 shows the dependences of $v_f$ on the concentration of NaCl in the presence of 0, 0.5, 2.5, and 10 mM KCl. The
values of $v_f$ at various KCl concentrations increased with increase in the concentration of NaCl, reached a maximum level, and then decreased with further increase in NaCl concentration, as already described for the case in 0 mM KCl. The curves given in this figure were calculated according to the following equation:

$$v_f = \frac{V_f}{1 + \frac{\phi_A}{[Na^+]}}$$

The observed values of $v_f$ approximately fitted this equation. As shown in Fig. 18, all the kinetic parameters, $V_f$, $\phi_A$, and $\phi_I$, decreased with increase in the concentration of KCl, and their dependences of KCl concentration under the conditions used were given by the following equations:

$$V_f = 8 + \frac{48}{1 + \frac{[Na^+]}{0.6 \text{ mM}}}, \quad (8),$$

$$\phi_A = \frac{10.3 \text{ mM}}{1 + \frac{[K^+]}{0.6 \text{ mM}}}, \quad (9),$$
and \( \phi_i = \frac{32 \text{ mM}}{[K^+] + \frac{1}{2.7 \text{ mM}}} \) \hspace{1cm} (10).

But as seen in this figure, the experimental values did not always agree well with the above equations.

Figure 19 shows the dependence of the value of \( v_f \) on concentration of KCl in the presence of 10 mM NaCl. The values of \( v_f \) at various concentrations of KCl in 10 mM NaCl were similar to those given in Fig. 17.

The dependence of \( v_f \) on NaCl concentration was also measured in the presence of 4 \( \mu \text{M} \) ATP, instead of 40 \( \mu \text{M} \) ATP (Figs. 16-19), since Kanazawa et al. (24) reported that ATP functions not only as substrate but also as a modulator for \( E_2 \text{ATP-formation} \). Figure 20 shows the dependence of the value of \( v_f \) on the concentration of NaCl in the presence of 4 \( \mu \text{M} \) ATP and absence of KCl. The value of \( v_f \) was about 1 mole/10^7 g protein/sec with 0.5 mM NaCl, and increased with
increase in the concentration of NaCl, reached a maximum value of 10 moles/10⁷ g protein/sec with 10 mM NaCl, and then decreased with further increase in the concentration of NaCl to about 0.5 moles/10⁷ g protein/sec with 600 mM NaCl. This pattern was similar to that obtained using 40 μM ATP (Fig. 16), though the values of \( v_f \) were less.

Figure 21 shows the dependences of the values of \( v_f \) with 4 mM ATP on the concentration of NaCl in the absence of KCl and in the presence of 0.5 mM KCl. The value of \( v_f \) reached a maximum with 10 mM NaCl in both cases. The maximum value in the absence of KCl was about 10 moles/10⁷ g protein/sec, and that in the presence of 0.5 mM KCl was about 7 moles/10⁷ g protein/sec. Thus, unlike in 40 μM ATP (Fig. 17), the maximum value of \( v_f \) in the absence of KCl was slightly higher than that in the presence of 0.5 mM KCl.

Discussion on the effect of Na⁺ and K⁺ on the partial reactions.—The results on the effects of Na⁺ and K⁺ on the rate of E₂ATP-formation (eqs. (7)-(10)) can be interpreted quantitatively by postulating two kinds of cooperative cation binding sites, as shown in Fig. 22: E₂ATP-formation
requires the binding of Na\(^+\) to an Na\(^+\)-site, of which the dissociation constant is 10.3 mM. The Na\(^+\)-binding to this site induces cooperative K\(^+\)-binding to a K\(^+\)-site, of which the dissociation constant is 0.6 mM, and this K\(^+\)-binding decreases the value of \(V_f\) to a low level and produces a decrease in the value of \(\phi_A\). On the other hand, the binding of Na\(^+\) to and Na\(^+\)-site, of which the dissociation constant is 32 mM, inhibits E\(_2\)ATP-formation. This binding of Na\(^+\) induces cooperative K\(^+\)-binding to a K\(^+\)-site of which the dissociation constant is 2.7 mM, and this K\(^+\)-binding decreases the value of \(\phi_I\). Furthermore, the cooperative binding of K\(^+\) was modulated by ATP, as shown in Figs. 17 and 21. E\(_2\)ATP-Formation was accelerated by 0.5 mM KCl in 40 \(\mu\)M ATP (Fig. 17), while it was slightly inhibited by 0.5 mM KCl in 4 \(\mu\)M ATP (Fig. 21).

The dependence on the Na\(^+\) concentration of \(1 + K_3\) showed rather a complicated behaviour as the concentration of K\(^+\) was varied (Fig. 15), although generally speaking, it increased with increase in the K\(^+\) concentration. More detailed studies are needed to obtain quantitative information on what changes in the affinity for cations occur when E\(_2\)ATP changes to EP.

The effects of Na\(^+\) and K\(^+\) on EP-decomposition (eq. (5)) can be interpreted by postulating three kinds of cation binding site (Fig. 22). The EP-decomposition is accelerated by K\(^+\) binding to a K\(^+\)-site, of which the dissociation constant is 0.043 mM. It is inhibited by two kinds of Na\(^+\) binding, one of which occurs at relatively low Na\(^+\) concentrations, although
this Na\(^+\) binding is competitively inhibited by much lower concentrations of K\(^+\). Two moles of Na\(^+\) per ATPase active site bind cooperatively to the other Na\(^+\)-site, and the dissociation constant of this binding is 190 mM, and the binding is not suppressed by K\(^+\).

Figure 22 shows a schematic representation of the interactions between ATPase and cations obtained from the kinetic studies on the elementary step. The Na\(^+\)- and K\(^+\)-bindings are related to each other. This complicated interrelation may be the structural basis for regulation of the Na\(^+\)-K\(^+\)-dependent ATPase reaction. Another feature to be pointed out is that the dissociation constants of Na\(^+\) at the step of EP\(_p\)-decomposition are larger than those of E\(_2\)ATP\(_2\)-formation. On the contrary, the dissociation constants of K\(^+\) at the step of E\(_2\)ATP\(_2\)-formation are larger than those of EP\(_p\)-decomposition. It remains to be clarified at which step these shifts in affinity for cations occur. However, it seems reasonable to conclude that the shifts in affinity already occur at the state of E\(_2\)ATP, since the equilibrium between E\(_2\)ATP and EP shifts toward E\(_2\)ATP on adding low concentrations of K\(^+\), even in the presence of high concentrations of Na\(^+\).

Properties of the Step from an Enzyme-ATP Complex to a phosphorylated Intermediate in the Reaction of Na\(^+\)-K\(^+\)-dependent ATPase (48)
The Equilibrium Step between E₂ATP and EP at Low Na⁺ Concentration—The existence of step 3 was first proposed (24) from the observations that the ratio of the steady-state rate of ATP-hydrolysis to the concentration of EP, \( v_0/[EP] \), was larger than the first order decay constant of EP, \( k_d \).

According to our reaction mechanism, the reciprocal of the equilibrium constant, \( K_3 \), at step 3 is given as \( v_0/([EP] \cdot k_d) - 1 \). The value of \( 1 + K_3 \) increased from 1.15 with increase in the KCl concentrations from 0 to 1 mM (24).

As described in the first and the second sections, the existence of the equilibrium step between E₂ATP and EP in the presence of high concentrations of NaCl was further demonstrated (42, 43, 47) by the following four types of experiments. First, the time-course of EP-disappearance was proportional to Pi-liberation after stopping EP-formation by adding EDTA, but the amounts of Pi liberated were always larger than those of decrease in EP, and the ratio was equal to the ratio of \( v_0/[EP] \) to \( k_d \), i.e., \( 1 + K_3 \). Second, the amount of EP decreased with increase in the concentration of KCl, but \([EP] (1 + K_3)\), which represents the total phosphorylation site of the enzyme, was always constant, irrespective to the concentration of KCl. Third, when KCl was added together with EDTA to EP, a fraction of EP decayed rapidly, and then the remaining EP decreased following first order kinetics. If we assume that the initial rapid decay in EP is due to a shift of the equilibrium toward E₂ATP on adding KCl, we can calculate
the value of $K_3$ in the presence of KCl. The value of $1 + K_3$ thus obtained was in agreement with the ratio of $v_0/\left[EP\right]$ to $k_d$. Fourth, the most conclusive evidence is the formation of ATP accompanied by the initial rapid decay of EP on adding KCl and EDTA (42,43). The amount of ATP formed was equal to the amount calculated from the change in $K_3$ on adding KCl, which was obtained by the methods mentioned above.

As described in the second section (47), the values of $1 + K_3$, deduced as $v_0/\left([EP] \cdot k_d\right)$, in the presence of KCl were much larger in the presence of low concentrations of NaCl than those in the presence of high concentrations of NaCl. For example, in the presence of 0.5 or 10 mM KCl, the value of $1 + K_3$ was about 12 at low concentrations of NaCl. This large value of $K_3$ prompted us to re-examine the existence of the equilibrium at low concentrations of NaCl.

Figure 23 shows the time-courses of $^{32}\text{P}_1$-liberation

Figure 23

and $E^{32}\text{P}$-disappearance after adding EDTA or unlabeled ATP to stop the formation of $E^{32}\text{P}$ in the presence of 10 mM NaCl and 0.4 mM KCl. The reaction was initiated by adding 40 $\mu$M $AT^{32}\text{P}$ to 0.29 mg/ml enzyme, and 0.3 sec after initiating the reaction, 20 mM EDTA or 4 mM unlabeled ATP was added to stop $E^{32}\text{P}$-formation. The time-courses of $^{32}\text{P}_1$-liberation
and \( E^{32P} \)-disappearance after adding EDTA were equal to those after adding unlabeled ATP. The amount of \( ^{32}P_1 \)-liberation was always much higher than that of \( E^{32P} \)-disappearance. For example, the amount of \( ^{32}P_1 \) liberated after a 1.5 sec reaction was about 8.5 moles/\( 10^7 \) g protein, while the amount of decrease in \( E^{32P} \) was 0.7 mole/\( 10^7 \) g protein. This suggests that the value of \( 1 + K_3 \) was about 12. However, the proportionality between \( P_1 \)-liberation and decrease in \( E^P \)-concentration after stopping \( E^P \)-formation was not satisfied in 10 mM NaCl. The latter reached the maximum level in 0.8 sec, but the former continued for 1.5 sec.

The rapid decrease in \( E^P \) during the initial phase after adding KCl together with EDTA to the reaction mixture was observed in the presence of low concentrations of NaCl. Figure 24 shows the initial phases of disappearance of \( E^P \).

Figure 24

after a KCl-jump in the presence of 5, 40, and 400 mM NaCl. The reaction was initiated by adding 40 \( \mu \)M ATP to 0.29 mg/ml enzyme. Three sec after initiating the reaction, 20 mM EDTA containing 0.5 mM KCl was added, and the time-course of \( E^P \)-disappearance was measured. Figure 24 shows the logarithm of the relative value of \( E^P \) plotted against the time after addition of EDTA-KCl.
From the value of $1 + K_3$ estimated as $v_0/([EP]k_d)$ in the absence of KCl reported in the second section (47) and the amount of rapid decay of EP after the KCl-jump given in Fig. 24, we calculated the value of $1 + K_3$ in the presence of 0.5 mM KCl. Table I summarized the values of $1 + K_3$ calculated as mentioned above and those calculated as $v_0/([EP]\cdot k_d)$ with 5, 40, 400 mM NaCl in the presence of 0.5 mM KCl. The values of $1 + K_3$ obtained by the two methods were similar. Thus, the existence of the equilibrium between $E_2$ATP and EP in the presence of a low concentration of NaCl was supported by the agreement of the values of $1 + K_3$ obtained from the three different methods.

Figure 25 shows the time-courses of EP-disappearance, $P_i$-liberation, and the amount of ATP remaining in the reaction mixture after a KCl-jump. The reaction was initiated by the addition of 0.11 μM ATP to reaction mixture containing 2.26 mg/ml enzyme and 20 mM NaCl at 15°. Two sec after initiating the reaction, solutions of 50 mM EDTA with or without 0.8 mM KCl were added, and the time-courses of EP-disappearance,
Pi-liberation, and the amount of ATP remaining in the reaction mixture were measured. As seen in this figure, the amount of ATP in the reaction mixture increased slightly but definitely when EDTA containing KCl was added to EP, but it decreased when KCl-free EDTA was added to EP. The amount of ATP formed by the KCl-jump was less than the value expected from the values of $1 + K_3$ described in the second section (47), and a larger fraction of EP-decrease was observed as Pi-liberation. It seemed that $K^+$ has more effect on EP-decomposition than on shift of the equilibrium in the presence of low concentrations of NaCl at 15°. This conjecture is consistent with the result shown in Fig. 23 that EP-disappearance reached a maximum level more rapidly than Pi-liberation did after adding EDTA-KCl.

Figure 26 shows the time-course of ATP-formation after a KCl-jump at 0°. At 3.5 sec after initiating the reaction, 50 mM EDTA with or without 0.8 mM KCl was added to EP under the same conditions as for Fig. 25, except that the temperature was 0°. The amount of ATP remaining in the reaction mixture (0.35 mole/10^8 g protein) after the addition of KCl-free EDTA did not change within 1 sec. On the other hand, a considerable amount of ATP was formed when EDTA-KCl was added to EP. The net amount of ATP formed reached a
maximum value of 0.65 mole/10^8 g protein, 0.4 sec after
the KCl-jump.

Thermodynamic Functions of the Step, \( E_2ATP \rightleftharpoons EP \). To clarify
the coupling mechanism between active transport of cations
and the enzyme reaction, it is very important to know the
thermodynamic functions of the step \( E_2ATP \rightleftharpoons E_p - ADP \), since
Kanazawa et al. (34) and Sumida et al. (35) showed that
the translocation of Ca^{2+} ions across the membrane of the
sarcoplasmic reticulum occurs in this step. In the present
study, we measured the temperature dependence of the equilibrium
constant, \( K_{eq} \), i.e. \( K_3^{-1} \), of step 3 to obtain the thermodynamic
functions in the presence of a high concentration of NaCl
and in the absence of KCl. Figure 27 shows the time-courses
of \( P_i \)-liberation and EP-disappearance after adding EDTA to

\[ \text{Figure 27} \]

stop EP-formation at 10°, 18°, and 25°. EP-Formation was
initiated by adding 0.18 \( \mu \)M ATP to 0.53 mg/ml enzyme in the
presence of 140 mM NaCl. At 0.3 sec after initiating EP-
formation, 50 mM EDTA was added, and the amounts of \( P_i \)
liberated and EP were measured. Although results were not
very accurate, the amount of \( P_i \)-liberation was more or less
proportional to decrease in the EP concentration after the
addition of EDTA. The curves in the figure were drawn
assuming that the proportional coefficients of \( P_i \)-liberation/
Ep-decrease at 10°, 18°, and 25° were 1.8, 1.7, and 1.5, respectively. We estimated the values of $K_{eq}$ at the three temperatures using these coefficients, and calculated the standard free energy change, $\Delta G^o$, at step 3, as $-RT \ln K_{eq}$.

Figure 28 shows the temperature dependence of the value of $\Delta G^o$. From the temperature dependence of $\Delta G^o$, we estimated the enthalpy change, $\Delta H^o$, and the entropy change, $\Delta S^o$, of step 3. At 5° both the rates of P$_i$-liberation and EP-disappearance were too small to be accurately determined, while at 30° they were too large to be accurately measured by the simple mixing apparatus. Therefore, the range of temperature used was rather narrow (10°-25°), and we could not accurately determine the values of $\Delta H^o$ and $\Delta S^o$. However, they were roughly estimated to be +4-5 Kcal mole$^{-1}$ and +15-16 entropy units mole$^{-1}$, respectively.

Recently, the existence of a reversible equilibrium between the second myosin-ATP complex and the reactive myosin-P-ADP complex was demonstrated (36,37) in the reaction of myosin-ATPase. In this case, the value of $\Delta H^o$ was +3 - +4 Kcal mole$^{-1}$, and the value of $\Delta S^o$ was +15 - +20 entropy units mole$^{-1}$(37). It is well known that phosphorylated enzyme of an acyl-phosphate type is obtained as an intermediate of the transport ATPase (1,2,4-19,25) by adding TCA to stop the
reaction, while $P_i$ is released by adding TCA to the reactive myosin-P-ADP complex of myosin ATPase (25). However, the finding that the thermodynamic parameters of the step in $Na^+K^+$-dependent ATPase between the second enzyme-ATP complex and the phosphorylated intermediate were similar to those of the step in myosin ATPase between the second myosin-ATP complex and the reactive myosin-P-ADP complex suggests the similarity of the chemical and energetic structures of phosphorylated intermediate in the transport ATPase and the reactive myosin-P-ADP complex in the contractile ATPase before the addition of TCA.

**SUMMARY**

The existence of two kinds of high energy phosphorylated intermediate, EP, with and without bound ADP, in the reaction of $Na^+K^+$-dependent ATPase was indicated by the following results.

1. Only one type of EP, i.e., that for which decay is unaffected by the addition of ADP, was observed in the presence of high concentrations of $Mg^{2+}$. On adding $K^+$ ($K^+$-jump) a fraction of the EP decayed rapidly, and then the time course of EP decomposition followed first-order kinetics. The initial rapid decay of EP by a $K^+$-jump was accompanied by the formation of an equal amount of ATP. The amount of ATP formed was equal to that calculated on the basis of the mechanism previously proposed by Kanazawa et al., that the equilibrium between an enzyme-ATP complex and EP shifts
toward formation of the enzyme-ATP complex on adding $K^+$. 

2. When NEM-treated enzyme was used in the presence of low concentrations of $Mg^{2+}$, two types of EP were observed. One was sensitive, and the other was insensitive to ADP. The decay of the former type was accelerated by the addition of ADP. The percentage of ADP-sensitive EP was zero in the initial phase of the reaction after adding ATP, and increased with time to a steady-state level.

3. These two types of experimental results support the following reaction mechanism for the $Na^+-K^+$-dependent ATPase:

$$E + ATP \rightleftharpoons E_1\cdot ATP \rightleftharpoons E_2\cdot ATP \rightleftharpoons E^{''}_{ADP} \rightleftharpoons E + P_i + ADP.$$ 

The two types of EP, with and without bound ADP, are both high energy forms. They are formed via two kinds of enzyme substrate complex, $E_1\cdot ATP$ and $E_2\cdot ATP$, and $E^{''}_{ADP}$ is in $K^+$-dependent equilibrium with $E_2\cdot ATP$.

On the basis of the reaction mechanism mentioned above, the effects of $Na^+$ and $K^+$ on the elementary steps in the reaction of $Na^+-K^+$-dependent ATPase were investigated in 0.5-600 mM NaCl and 0-10 mM KCl at a fixed concentration (1 mM) of MgCl$_2$, and the following results were obtained:

4. The rate of $E_2\cdot ATP$-formation, $v_f$, increased with increase in the $Na^+$ concentration, reached a maximum level, and then decreased further increase in the $Na^+$ concentration at various $K^+$ concentrations. The value of $v_f$ was given as
5. The reciprocal of the equilibrium constant, $K_3$, of the step $E_1\cdot ATP \rightleftharpoons E_2\cdot ADP$ in the presence of low concentrations of $Na^+$ was larger than that in the presence of high concentrations of $Na^+$, indicating that the equilibrium shifted markedly toward $E_2\cdot ATP$ at low concentrations of $Na^+$. The relation of $K_3$ with $Na^+$ concentration was rather complicated on varying the concentration of $K^+$. However, generally speaking, it increased with increase in the $K^+$ concentration.

6. The $EP$-decomposition was markedly activated by even low concentrations of $K^+$, and inhibited by high concentrations of $Na^+$. The inhibition by $Na^+$ was partially suppressed by $K^+$. The rate constant of $EP$-decomposition, $v_o/\lbrack EP\rbrack$, was given by

$$
v_o/\lbrack EP\rbrack = \frac{18.4/(1 + \frac{0.043 \text{ mM}}{[K^+]})}{1 + \frac{[Na^+]}{130 [K^+]} + \frac{[Na^+]}{(190 \text{ mM})^2}} \text{ (sec}^{-1})$$
where \((v_0/\text{[EP]})_{K^+0}\) was the value of \(v_0/\text{[EP]}\) in the absence of \(K^+\).

Next, some kinetic and thermodynamic properties of the step \(E_2'\text{ATP} \rightleftharpoons E_2'\text{ADP} \) were investigated, and the following results were obtained:

7. The existence of quasi-equilibrium between \(E_2'\text{ATP}\) and \(E_2'\text{ADP}\) in the presence of low concentrations of \(Na^+\) was suggested from the fact that the values of the reciprocal of the equilibrium constant, \(K_3\), of the step \(E_2'\text{ATP} \rightleftharpoons E_2'\text{ADP}\) obtained by the following three methods were almost equal. a) The value of \(1 + K_3\) was estimated as the ratio of \(v_0/\text{[EP]}\) to \(k_d\), where \(v_0\) was the rate of ATP-hydrolysis in the steady-state, and \(k_d\) the first order rate constant of EP-disappearance after stopping the EP-formation. b) It was calculated from the ratio of the amount of \(P_i\) liberated and that of decrease in EP after stopping the EP-formation. c) The value of \(K_3\) was also calculated from the initial rapid decrease in EP on adding \(K^+\) and EDTA, assuming that the rapid decrease was due to shift of the equilibrium toward \(E_2'\text{ATP}\) on adding \(K^+\). Although ATP-formation due to shift of the equilibrium toward \(E_2'\text{ATP}\) by a \(K^+-\)jump in the presence of a low concentration of \(Na^+\) was observed at \(0^\circ\text{C}\), the amount of ATP formed by a \(K^+-\)jump at \(15^\circ\text{C}\) was less than the value expected from shift of the equilibrium.

8. The values of \(\Delta H^\circ\) and \(\Delta S^\circ\) of the step \(E_2'\text{ATP} \rightleftharpoons E_2'\text{ADP}\) were estimated in the presence of a sufficient amount of \(Na^+\) and the absence of \(K^+\). They were \(+4 - 5\) Kcal mole\(^{-1}\) and 15-16 entropy units mole\(^{-1}\), respectively.
ACKNOWLEDGEMENTS

The author wishes to thank Professor Yuji Tonomura for his valuable suggestions and continuous encouragements. This work was supported by grants from the Ministry of Education, from the Muscular Dystrophy Associations of America, Inc.
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Table I  Comparison of Values of $1 + K_3$ Determined as $v_0/([EP]; k_d)$ with Those Estimated from Rapid Decay of EP after Adding K$^+$. 

The reaction was performed using 0.29 mg/ml enzyme and 40 mM $\gamma$-32P-ATP in the presence of various concentrations of NaCl under the conditions described in text. Three sec after initiating the reaction, TCA was added to the reaction mixture and the values of $v_0$ and [EP] were measured. Three sec after initiating the reaction, 20 mM EDTA containing 0.5 mM KCl was added to the reaction mixture, and the value of $k_d$ and the amount of rapid decay in EP during the initial phase were measured (cf. Fig. 24) 

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>$v_0/([EP]; k_d)$</th>
<th>Rapid decay of EP after adding K-EDTA</th>
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<tbody>
<tr>
<td>5</td>
<td>11.3</td>
<td>8.5</td>
</tr>
<tr>
<td>40</td>
<td>3.1</td>
<td>3.0</td>
</tr>
<tr>
<td>400</td>
<td>1.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>
LEGENDS FOR FIGURES

Fig. 1. Effect of ADP on decay of EP formed in the presence of a high concentration of Mg$^{2+}$. The phosphorylation reaction was started by addition of 0.05 ml of 0.2 mM $\text{AT}^{32}\text{P}$ to 0.85 ml of reaction mixture, to give final concentrations of 5 mM MgCl$_2$, 100 mM NaCl and 0.14 mg/ml enzyme at pH 7.5 (30 mM histidine buffer) and 0°C. Two and 0.2 sec after starting the phosphorylation reaction, 0.1 ml of 0.5 M EDTA-Tris (pH 7.5) ($\times$, $\oplus$) or 31 mM ADP containing 27 mM MgCl$_2$ (pH 7.5 with Tris) ($\circ$, $\bullet$) was added. The final volume of the reaction mixture was 1 ml. Phosphorylation time: $\circ$, $\times$, 2 sec; $\bullet$, $\oplus$, 0.2 sec.

Fig. 2. Formation of ATP on adding K$^+$ to EP formed in the presence of a high concentration of Mg$^{2+}$. The phosphorylation reaction was started by addition of 0.05 ml of 2 mM $\text{AT}^{32}\text{P}$ to 0.85 ml of reaction mixture, to give final concentrations of 5 mM MgCl$_2$, 100 mM NaCl and 3.5 mg/ml enzyme at pH 8.5 (100 mM Tris-HCl) and 15°C. Two sec after starting the phosphorylation reaction, TCA was added, or 0.1 ml of 0.5 M EDTA-Tris containing 7.7 mM KCl (pH 8.5) was added first, and 0.5 sec later TCA was added. The final volume of the reaction mixture was 1 ml. The two samples were centrifuged and $\text{P}_i$ was removed from the supernatants by the method of Martin and Doty. Nucleotides were adsorbed on charcoal, extracted with NH$_4$OH-ethanol (1 % : 50 %), and charged on a DEAE-Sephadex column (1.8 x 18 cm). The column was eluted with
a gradient of 0 to 0.5 M KCl in 0.1 M Tris-Cl (pH 8.3). Fractions of 5 ml were collected. optical density at 260 nm; radioactivity of sample without added K⁺; radioactivity of sample with added K⁺. The background count was not subtracted.

Fig. 3. Time-course of ATP-formation after adding K⁺ to EP formed in the presence of a high concentration of Mg²⁺. The phosphorylation reaction was performed as described for Fig. 2, except that final concentrations of 0.05 µM ATP, 150 mM NaCl and 1.63 mg/ml enzyme were used. Two sec after starting the reaction, 0.1 ml of 0.5 M EDTA-Tris (pH 8.5) (X) or 0.5 M EDTA-Tris containing 7.7 mM KCl (pH 8.5) (○, ●, △) was added. The amounts of EP and ATP formed were measured as described in the text. X, ○, amount of EP. △, amount of total ATP. ●, amount of ATP formed.

Fig. 4. Effect of ADP on the decay of EP formed in the presence of a low concentration of Mg²⁺. The phosphorylation reaction was started by addition of 0.05 ml of 0.1 mM ATP to 0.85 ml of reaction mixture to give final concentration of 10 µM MgCl₂, 140 mM NaCl and 0.156 mg/ml enzyme at pH 7.5 (30 mM histidine buffer) and 15°C. One sec after starting the reaction, 0.1 ml of 4.5 mM Mg-ADP (●) or Mg-unlabeled ATP (pH 7.5 with Tris)(○) was added. The final volume of the reaction mixture was 1 ml.
Fig. 5. Effect of ADP on the decay of EP with NEM-treated enzyme in the presence of a low concentration of Mg

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The enzyme (0.68 mg/ml) was exposed to 1 mM NEM for 1 hr at pH 8.4 (0.1 M Tris-HCl) and 15°C. The phosphorylation reaction was started by adding 0.05 ml of 0.2 mM AT\textsuperscript{32}P to 0.85 ml of reaction mixture to give final concentrations of 10 \mu M MgCl\textsubscript{2}, 100 mM NaCl and 0.135 mg/ml enzyme at pH 7.5 (30 mM histidine buffer) and 0°C. One sec after starting the reaction, 0.1 ml of 0.5 M EDTA-Tris (pH 7.5) (\times), 0.5 M EDTA-Tris containing 1 mM KCl (\bigcirc) or 10 mM Mg-ADP (\bullet) was added. The final volume of the reaction mixture was 1 ml.

Fig. 6. Effects of ADP on EP-decay at various time after starting the phosphorylation reaction with NEM-treated enzyme in the presence of a low concentration of Mg\textsuperscript{2+}. The phosphorylation reaction was performed as for Fig. 5, using NEM-treated enzyme. At intervals after starting the reaction, 0.1 ml of 0.5 M EDTA-Tris (pH 7.5) (\times) or 31 mM ADP containing 27 mM MgCl\textsubscript{2} (pH 7.5 with Tris) (\bigcirc) was added (\downarrow). The final volume of the reaction mixture was 1 ml. \bullet, amount of EP formed; \bigcirc, amount of EP after adding Mg-ADP (\bigcirc) or EDTA (\times).

Fig. 7. Time-course of formation of ADP-sensitive EP with NEM-treated enzyme in the presence of a low concentration of Mg\textsuperscript{2+}. The ratio of the amount of ADP-sensitive EP, \[\text{EP}]_{\text{ADP}},
to the total amount of EP, \([EP]_{\text{total}}\), was calculated as described in the text from the results shown in Fig. 6, and is plotted against time. ●, amount of total EP formed; △, ratio of amount of ADP-sensitive EP to that of total EP.

Fig. 8. Dependence of the rate constant of EP-decomposition on the concentration of NaCl in the absence of KCl. 0.29 mg/ml enzyme, 4 μM \(AT^{32}P\), 1 mM MgCl₂, 0.5-400 mM NaCl, 100 mM Tris-HCl, pH 8.5, 15°. The reaction was stopped after 3 sec by adding TCA, and the amounts of \(P_i\) liberated and EP formed were measured. ●, concentration of EP; ○, rate of ATP-hydrolysis, \(v_0\); △, rate constant of EP-decomposition calculated as \(v_0/\text{[EP]}\).

Fig. 9. Dependence of the rate constant of EP-decomposition on the concentration of NaCl in the presence of 2.5 mM KCl. Experimental conditions were as described for Fig. 8, except that the concentration of KCl was 2.5 mM and that the concentration of \(AT^{32}P\) was 40 μM. ●, concentration of EP; ○, rate of ATP-hydrolysis, \(v_0\); △, rate constant of EP-decomposition calculated as \(v_0/\text{[EP]}\).

Fig. 10. Dependences of the rate constants of EP-decomposition on the concentration of NaCl at various concentrations of KCl. Experimental conditions were as described for Fig. 8, except that the concentration of \(AT^{32}P\) was 40 μM and that the concentrations of KCl were 0 (△), 0.1 (○), 0.5 (●), 2.5 (△), and 10 mM (▲).
Fig. 11. Dependence of the reciprocal of the equilibrium constant, $K_3$, at the step $E_2 \text{ATP} \rightleftharpoons E\cdot ADP$ on the concentration of KCl in the presence of 10 mM NaCl. Experimental conditions were as described for Fig. 8, except that the concentration of $\text{AT}^{32}\text{P}$ was 40 $\mu$M, $\square$, first order decay constant of EP, $k_d$; $\times$, rate constant of EP-decomposition calculated as $v_0/[\text{EP}]$; $\sim \sim \sim$, $1 + K_3$.

Fig. 12. Dependence of the reciprocal of the equilibrium constant, $K_3$, of the step $E_2 \text{ATP} \rightleftharpoons E\cdot ADP$ on the concentration of NaCl in the absence of KCl. Experimental conditions were as described for Fig. 8. $\square$, $k_d$; $\times$, $v_0/[\text{EP}]$; $\sim \sim \sim$, $1 + K_3$.

Fig. 13. Dependence of the reciprocal of the equilibrium constant, $K_3$, of the step $E_2 \text{ATP} \rightleftharpoons E\cdot ADP$ on the concentration of NaCl in the presence of 2.5 mM KCl. Experimental conditions were as described for Fig. 8, except that the concentration of $\text{AT}^{32}\text{P}$ was 40 $\mu$M. $\square$, $k_d$; $\times$, $v_0/[\text{EP}]$; $\sim \sim \sim$, $1 + K_3$.

Fig. 14. Dependence of the reciprocal of the equilibrium constant, $K_3$, of the step $E_2 \text{ATP} \rightleftharpoons E\cdot ADP$ on the concentration of NaCl in the presence of 10 mM KCl. Experimental conditions were as for Fig. 8, except that the KCl concentration was 10 mM and the concentration of ATP was 40 $\mu$M. $\square$, $k_d$; $\times$, $v_0/[\text{EP}]$; $\sim \sim \sim$, $1 + K_3$. 
Fig. 15. Dependences of the reciprocals of the equilibrium constants, \( K_3 \) of the step \( E_2ATP \rightleftharpoons E_2A^P \) on the concentration of NaCl at various concentrations of KCl. Experimental conditions were as for Fig. 8, except that the ATP concentration was 40 \( \mu \)M and that the concentrations of KCl were 0 (- - - - ), 0.5 (---), 2.5 (---) and 10 mM (---).

Fig. 16. Dependence of the rate of \( E_2ATP \)-formation on the concentration of NaCl in the absence of KCl. 0.29 mg/ml enzyme, 40 \( \mu \)M \( AT^{32}P \), 1 mM MgCl<sub>2</sub>, 1-600 mM NaCl, 100 mM Tris-HCl, pH 8.5, 15°. The ATPse reaction was stopped by adding TCA at 0.1 sec after initiating the reaction. ⋄, observed rate of EP-formation, \( v_f \); ○, rate of \( E_2ATP \)-formation, \( v_f \), calculated as \( v_f = (1 + K_3) \).

Fig. 17. Dependences of the rates of \( E_2ATP \)-formation on the concentration of NaCl at various concentrations of KCl. Experimental conditions were as described for Fig. 16, except that the concentrations of KCl were 0 (O), 0.5 (∗), 2.5 (∆), or 10 mM (∆).

Fig. 18. Dependences of the kinetic parameters of the rate of \( E_2ATP \)-formation on the concentration of KCl. The dependence of the rate of \( E_2ATP \)-formation, \( v_f \), on NaCl and KCl concentrations was given by

\[
v_f = \frac{V_f}{1 + \frac{\Phi_A}{[Na^+]}} \frac{[Na^+]}{\Phi I} (\text{moles/10}^7 \text{ g protein/sec})
\]
\[
x, V_f; \bullet, \phi_A; \bigcirc, \phi_I. \quad \text{Curve are } V_f = 8 + \frac{48}{1 + \frac{[K^+]}{0.6 \text{ mM}}}.
\]

\[
\phi_A = \frac{10.3 \text{ mM}}{1 + \frac{[K^+]}{0.6 \text{ mM}}} \quad \text{and} \quad \phi_I = \frac{32 \text{ mM}}{1 + \frac{[K^+]}{2.7 \text{ mM}}}, \text{ respectively.}
\]

Fig. 19. Dependence of the rate of $E_2$ATP-formation on the concentration of KCl in the presence of 10 mM NaCl. Experimental conditions were as described for Fig. 16, except that the values of $v_f$, were measured at various concentrations of KCl and at a fixed concentration of NaCl (10 mM). ●, observed rate of EP-formation, $v_f$; ○, rate of $E_2$ATP-formation, $v_f$.

Fig. 20. Dependence of the rate of $E_2$ATP-formation on the concentration of NaCl in the absence of KCl at a low concentration of ATP. Experimental conditions were as for Fig. 16, except that 4 µM AT$^{32}$P was used. ●, observed rate of EP-formation, $v_f$; ○, rate of $E_2$ATP-formation, $v_f$.

Fig. 21. Dependence of the rate of $E_2$ATP-formation on the concentration of NaCl in the absence of KCl and that in the presence of 0.5 mM KCl at a low concentration of ATP. Experimental conditions were as for Fig. 16, except that 4 µM AT$^{32}$P, and 0 (○) or 0.5 mM KCl (●) were used.
Fig. 22. Schematic representation of cation sites of Na\(^+\)-K\(^+\)-dependent ATPase. For explanation see text.

Fig. 23. Time-courses of P\(_i\)-liberation and EP-disappearance after adding EDTA-KCl to EP. 0.29 mg/ml enzyme, 40 \(\mu\)M \(\text{AT}^{32}\text{P}\), 1 mM MgCl\(_2\), 10 mM NaCl, 0.4 mM KCl, 100 mM Tris-HCl, pH 8.5, 15\(^\circ\)C. At 0.3 sec after initiating the phosphorylation reaction, 20 mM EDTA (▲, △) or 4 mM unlabeled ATP (●, ○) was added to EP. ▲, ●, EP-decrease; △, ○, \(\text{P}_i\)-liberation; — — — time-course of EP-disappearance calculated assuming that EP disappeared in parallel with \(\text{P}_i\)-liberation.

Fig. 24. Initial rapid decay in EP after adding EDTA-KCl. 0.29 mg/ml enzyme, 40 \(\mu\)M \(\text{AT}^{32}\text{P}\), 1 mM MgCl\(_2\), 100 mM Tris-HCl, pH 8.5, 15\(^\circ\). Three sec after initiating the phosphorylation reaction, 20 mM EDTA containing 0.5 mM KCl was added to EP. Concentrations of NaCl: ○, 5; ●, 40; □, 400 mM.

Fig. 25. ATP-Formation accompanying decrease in EP by a KCl-jump in the presence of a low concentration of NaCl at 15\(^\circ\). 2.26 mg/ml enzyme, 0.11 \(\mu\)M \(\text{AT}^{32}\text{P}\), 1 mM MgCl\(_2\), 20 mM NaCl, 100 mM Tris-HCl, pH 8.5, 15\(^\circ\). Two sec after initiating the phosphorylation reaction, 50 mM EDTA containing 0.8 mM KCl (○, ●, △) or KCl free EDTA (□) was added. ○, concentration of \(\text{P}_i\)-liberated; ●, concentration of EP; △, □, concentration of ATP.
Fig. 26. ATP-Formation from EP by a KCl-jump in the presence of a low concentration of NaCl at 0°. Experimental conditions were as described for Fig. 25, except that the temperature was 0°. EDTA-KCl (△) or KCl free EDTA (□) was added to EP at 3.5 sec after initiating the phosphorylation reaction.

Fig. 27. Time-courses of P_i-liberation and EP-disappearance after adding EDTA at various temperatures. 0.53 mg/ml enzyme, 0.18 μM ATP, 1 mM MgCl_2, 140 mM NaCl, 100 mM Tris-HCl, pH 8.5. At 0.3 sec after initiating EP-formation, 50 mM EDTA was added and the time-courses of P_i-liberation and EP-decrease were measured. Temperature of EP-decrease: ◆, 10°; △, 18°; ■, 25°. Temperature of P_i-liberation: ○, 10°; △, 18°; □, 25°. Curves were drawn assuming that P_i-liberation was proportional to EP-decrease.

Fig. 28. Temperature dependence of the standard free energy change of step E_2ATP ⇌ E_2∼ADP. The equilibrium constant, K_{eq}, was calculated from the proportionality of P_i-liberation and EP-decrease shown in Fig. 27, and ΔG° was obtained as -RT ln K_{eq}. 
Fig. 8

The graph shows the concentration of NaCl (mM) on the x-axis and the [EP] (molecules/10^7 g protein) on the y-axis. Two curves are plotted, one solid and one dashed, representing different conditions or measurements. The dashed curve crosses the solid curve at a higher concentration of NaCl, indicating a change in the reaction or process under study.

[Diagram with axes labeled: CONCENTRATION OF NaCl (mM) on the x-axis and [EP](molecules/10^7 g protein) on the y-axis. Two curves, one solid and one dashed, are plotted.]
CONCENTRATION OF NaCl (mM)
Fig. 12

CONCENTRATION OF NaCl (mM)

k_d (sec^{-1}) (□)

1 + K_3 (- - -)

v_0 / [EP] (sec^{-1}) (x)

0

0.5

1.0

1.5

2.0

2.5

3.0

0

1

10

100

0
Fig 16

CONCENTRATION OF NaCl (mM)

$V_f$ (moles EP/107 g protein/sec) (C)

$V_f$ (moles EP/107 g protein/sec) (O)
CONCENTRATION OF KCl (mM)
CONCENTRATION OF KCl (mM)
CONCENTRATION OF NaCl (mM)
Fig. 21

CONCENTRATION OF NaCl (mM)

$V_f$ (moles EP/106 g protein/sec)
(1) $E_2 \cdot $ATP FORMATION

$$v_f = \frac{v_f}{1 + \frac{[Na]}{[Na]} + \frac{[Na]}{[Na]}}$$

(10.3) Na$^+$ $\rightarrow$ Na$^+$(32)

(0.6) K$^+$ $\rightarrow$ K$^+$(2.7)

(2) $E_2^{ADP}$ DECOMPOSITION

$$k'_4 = k_4 / \left[ 1 + \frac{[Na]}{[Na]} + \left( \frac{[Na]}{[Na]} \right)^2 \right]$$

(0.043) K$^+$ $\rightarrow$ K$^+$(0.1)

Na$^+$(0.1)

(130:1): Dissociation Constant in mM
Fig 27

\[ \Delta[P_i](0, A, O, △, □) \text{ (moles/10}^7\text{g protein)} \]

\[ \text{REACTION TIME (sec)} \]