



Title	Phosphorylated Intermediates of Ca^{2+} , Mg^{2+} -Dependent ATPase of the Sarcoplasmic Reticulum from Skeletal Muscle
Author(s)	Takisawa, Haruhiko
Citation	大阪大学, 1979, 博士論文
Version Type	VoR
URL	https://hdl.handle.net/11094/24455
rights	
Note	

The University of Osaka Institutional Knowledge Archive : OUKA

<https://ir.library.osaka-u.ac.jp/>

The University of Osaka

Phosphorylated Intermediates of Ca^{2+} , Mg^{2+} -Dependent
ATPase of the Sarcoplasmic Reticulum from Skeletal Muscle

Haruhiko TAKISAWA

CONTENTS

Introduction	1
Part I. Factors affecting the transient phase of the Ca^{2+} , Mg^{2+} -dependent ATPase reaction of sarcoplasmic reticulum from skeletal muscle	10
Experimental procedure	11
Results	14
Discussion	27
References	35
Summary	37
Part II. ADP-sensitive and -insensitive phosphorylated intermediates of solubilized Ca^{2+} , Mg^{2+} -dependent ATPase of the sarcoplasmic reticulum from skeletal muscle	39
Experimental procedure	40
Results	45
Discussion	82
References	88
Summary	90
Acknowledgment	92

ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
AT ³² P	[γ - ³² P]ATP
ATPase	adenosine triphosphatase
C ₁₂ E ₈	octaethyleneglycol mono-n-dodecylether
CK	creatine kinase
CP	creatine phosphate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
EGTA	ethyleneglycol bis(β -aminoethylether)-N,N,N',N'- tetraacetate
EP	phosphorylated intermediate
FSR	fragmented sarcoplasmic reticulum
MalNEt	N-ethylmaleimide
PL	phospholipid
SR	sarcoplasmic reticulum
SSR	solubilized sarcoplasmic reticulum
TCA	trichloroacetic acid
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid

INTRODUCTION

The sarcoplasmic reticulum (SR), a highly differentiated endoplasmic reticulum in muscle cells, consists of continuous vesicles, tubules, and cisternae that forms a network surrounding the myofibrils. The tubular structure running transversely to the fiber axis is called the transverse tubule or T-system which is found to be continuous with the surface membrane (sarcolemma). These membrane systems play an important role in controlling muscle contraction.

When the sarcolemma is excited, the T-system mediates the transmission of the action potential to the interior of the fiber, thus causing localized activation of the SR. In turn, Ca^{2+} is released from SR, in which Ca^{2+} is normally stored in resting muscle, and thus induces muscle contraction. The subsequent re-accumulation of Ca^{2+} by the SR coupled with ATP hydrolysis (active transport of Ca^{2+}) causes the relaxation of muscle (1).

The active transport of Ca^{2+} across the SR membrane has been studied widely using fragmented SR (FSR) isolated from muscle homogenates as a microsomal fraction. Hasselbach and Makinose (2) and Ebashi and Lipmann (3) first demonstrated that the microsomal fraction was capable of removing a significant amount of Ca^{2+} from the medium in the presence of ATP and Mg^{2+} . Hasselbach and Makinose (2,4,5) also reported that FSR shows Ca^{2+} , Mg^{2+} -dependent ATPase activity. Furthermore, they demonstrated that 2 moles of Ca^{2+} were transported into the vesicles when 1 mol of ATP was hydrolyzed, and FSR could produce a 1000- to 5000-fold Ca^{2+} gradient across the membrane.

FSR isolated from skeletal muscle shows empty vesicles about 80-100 nm in diameter. The surface of the vesicle is covered with particles about 4 nm in diameter which are considered to be a part of the ATPase molecules (6,7). MacLennan (8) and MacLennan et al. (9) purified the ATPase of FSR solubilized with deoxycholate by stepwise fractionation with ammonium acetate. The purified ATPase preparation consists of a major protein of the ATPase with a molecular weight of about 100,000 daltons and various kinds of phospholipids. This preparation reforms membrane on removal of detergent. Electron-microscopic observations revealed that the reformed membrane exhibited structure similar to the intact membrane. The purified ATPase catalyzed a Ca^{2+} , Mg^{2+} -dependent ATP hydrolysis. However, the reformed membrane from the purified ATPase is incapable of accumulating Ca^{2+} . Recently, reconstitution of functional vesicles from purified ATPase and phospholipids was achieved by many investigators (6,7). These findings indicate that the active transport of Ca^{2+} through the SR membrane requires only two components, the ATPase protein and phospholipids.

FSR have been proved suitable material for studying molecular mechanism of active cation transport, because of its many distinct characteristics: (1) Easy preparation of FSR and a high content of the ATPase; (2) Tightly coupled ATP hydrolysis and Ca^{2+} transport; (3) A clear distinction of the sidedness of the FSR membrane; (4) Easy control of the Ca^{2+} and Mg^{2+} concentration in the medium by the use of the chelating agents like EGTA and EDTA; (5) Easy destruction of the membrane structure without loss of the ATPase activity by some detergents, and (6) Complete reversibility of the active transport process. These favorable features of this

system have led, in recent years, to rapid and detailed developments in studies on the molecular mechanism of coupling between Ca^{2+} transport and the ATPase reaction.

Hasselbach and Makinose (4,5) and Ebashi and Lipmann (3) found that FSR catalyzed a rapid phosphate exchange between ADP and ATP which exhibited similar Ca^{2+} -dependency as found in the case of ATP hydrolysis and Ca^{2+} uptake by FSR. Thus, the existence of a high-energy phosphorylated intermediate was proposed as a component of the reaction mechanism. Yamamoto and Tonomura (10,11) and Makinose (12) found that a protein of FSR was phosphorylated when the Ca^{2+} , Mg^{2+} -dependent ATPase reaction of this membrane with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (AT^{32}P) was quenched by trichloroacetic acid (TCA). This finding was confirmed by Martonosi (13,14), Inesi and Almendares (15), and Inesi et al. (16). The maximum amount of phosphate incorporated was found about 1 mole per mole of the ATPase protein (9,17). Phosphoprotein levels at the steady state depended on the concentrations of Ca^{2+} and ATP and paralleled with the Ca^{2+} -dependent ATPase activity (10,14,16). These results strongly suggested that the phosphorylated protein is a true intermediate of the Ca^{2+} , Mg^{2+} -dependent ATPase reaction. This assumption was later proved when EP formation and its decomposition into $\text{E} + \text{P}_i$ were analyzed extensively. The time course of P_i liberation agreed well with that calculated from the observed time course of EP formation, assuming that EP was an intermediate in the reaction with a specific turnover rate (18).

The phosphoprotein isolated after quenching by TCA is stable at acidic pH, but unstable at alkaline pH (10,11,12). It is hydrolyzed by treatment with hydroxylamine (10,12), resulting in the formation of hydroxamate (19). These stability characteristics

indicate that this phosphoprotein is similar to the acylphosphoprotein intermediate of Na^+ , K^+ -dependent ATPase (20,21).

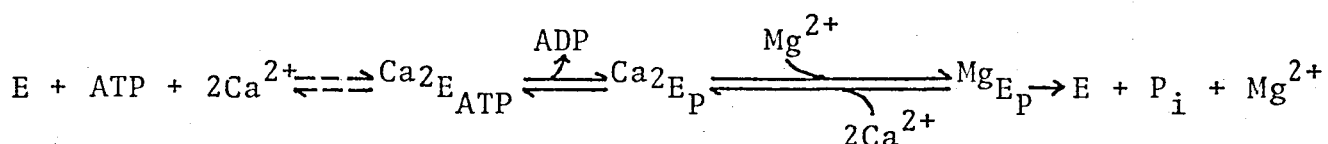
Bastide *et al.* (22) examined the chemical and electrophoretic properties of ^{32}P -labeled phosphoryl peptide produced after proteolysis of phosphorylated intermediates of Ca^{2+} , Mg^{2+} -dependent ATPase of FSR and Na^+ , K^+ -dependent ATPase of kidney microsomes, and found that the aspartyl residue was phosphorylated and the probable active site tripeptide sequence for both ATPases was (Ser or Thr)-Asp-Lys. Degani and Boyer (23) reported that the reductive cleavage of the acylphosphate bond of the phosphoprotein by sodium $[\text{}^3\text{H}]$ -borohydride yielded $[\text{}^3\text{H}]$ homoserine after acid hydrolysis of the protein, confirming that the phosphoryl group was covalently bound to the β -carboxyl group of aspartate.

Since the formation and decomposition of the EP intermediate can be measured kinetically, these two reaction steps have been studied extensively by many investigators. Kanazawa *et al.* (18) first measured the reaction of FSR in the presteady state using a simple rapid mixing apparatus, and showed that the time course of P_i liberation consisted of a lag phase, a burst phase and a steady phase, whereas EP was formed without a lag phase and its amount rapidly reached the steady-state level after the start of the reaction. More recently, Froehlich and Taylor (24) measured the time course of EP formation and P_i liberation using a rapid quenching method in detail. They observed an overshoot in the phosphorylation reaction and the P_i burst which coincided with the transient decay of EP when the reaction was started by adding ATP. They explained these observations by assuming that EP was rapidly hydrolyzed to an acid-labile phosphate intermediate (E·P) which was in equilibrium with EP. They also observed the initial

burst of P_i liberation by FSR ATPase even in the absence of Ca^{2+} (25). Since the time course of the P_i burst in the absence of Ca^{2+} was very similar to that of the P_i burst of the Ca^{2+} , Mg^{2+} -dependent ATPase reaction, they assumed the Ca^{2+} -independent reaction to be an alternate pathway of the enzyme. They proposed a flip-flop model in which the enzyme functions as a dimer for coupled transport of Ca^{2+} and Mg^{2+} .

The Ca^{2+} -dependent hydrolysis of ATP by FSR requires both Ca^{2+} and Mg^{2+} for full activation. The requirements of these divalent cations for each reaction steps i.g. the formation and decomposition of EP, have been studied by many investigators and it has been shown that the phosphorylation of the ATPase by Mg^{2+} -ATP requires Ca^{2+} and the decomposition of EP requires Mg^{2+} (6,7,26).

Kanazawa et al. (18) and Sumida and Tonomura (27) proposed the following reaction scheme which contains two kinds of EP, based on their kinetic studies of partial reactions of ATPase and also of the initial phase of Ca^{2+} accumulation:



Their kinetic studies were conducted in the presence of sufficient amounts of KCl. However, Shigekawa et al. (28) and Shigekawa and Dougherty (29,30) recently reported the existence of two kinds of EP in the reaction of purified SR ATPase in the absence of added alkali metal salts. One kind of EP could react with ADP to form ATP (ADP-sensitive EP) and the other could not (ADP-insensitive EP). They also reported that all the EP formed in the presence of sufficient amounts of added alkali metal salts could react with ADP to form ATP. At present, the relationship between two kinds

of EP proposed by Kanazawa et al. (18) and Sumida and Tonomura (27) and those reported by Shigekawa and co-workers (28,29,30) is not known.

The present thesis consists of Part I and II. Part I deals with factors affecting the transient phase of the Ca^{2+} , Mg^{2+} -dependent ATPase reaction of FSR. Froehlich and Taylor (24) proposed the existence of E·P as a new reaction intermediate in Ca^{2+} , Mg^{2+} -dependent ATPase reaction. However, the possibility that an appreciable amount of E·P exists in equilibrium with EP has recently been excluded by Sumida et al. (31). More recently, Kurzmack and Inesi (32) confirmed the existence of the initial P_i burst of the ATPase reaction both in the presence and absence of Ca^{2+} . According to them, the burst size of the Ca^{2+} -independent ATPase reaction at saturating substrate concentrations was about three times greater than the number of available enzymatic sites. This finding also shows clearly that the P_i burst of the Ca^{2+} -independent ATPase reaction is not caused by the formation of E·P as assumed by Froehlich and Taylor. In order to clarify the following two problems, I studied the reaction of Ca^{2+} , Mg^{2+} -dependent ATPase of FSR in the presteady state: (i) The discrepancy between the work of Kanazawa et al. (18) and Froehlich and Taylor (24) on P_i burst, and (ii) the possibility of the existence of E·P during the presteady state. I found that the P_i burst observed by Froehlich and Taylor (24) was due to a mechanism different from that of the P_i burst observed by Kanazawa et al. (18). My findings on marked changes in the reaction profiles during the transient phase with changes in the conditions for starting the reaction were difficult to explain with the Froehlich-Taylor mechanism, and could be

attributed to the heterogeneity of the initial enzyme state in the membrane. The latter conclusion was supported by the result obtained using solubilized ATPase, which showed neither the EP overshoot nor the P_i burst.

Kanazawa *et al.* (18), Sumida and Tonomura (27) and Shigekawa and co-workers (28,29,30) studied the reaction of Ca^{2+} , Mg^{2+} -dependent ATPase using the membrane preparation of FSR or purified ATPase. However, Ca^{2+} , Mg^{2+} -dependent ATPase in the SR membrane shows much more complicated kinetic properties than those of solubilized SR ATPase as described in Part I. Therefore, I studied the kinetic properties of Ca^{2+} , Mg^{2+} -dependent ATPase of solubilized SR (SSR), especially with the properties of two kinds of EP to clarify whether the two kinds of EP reported by Shigekawa and co-workers (28,29,30) exist in the presence of sufficient amounts of KCl or not, and how the two kinds of EP proposed by Shigekawa and co-workers (28,29,30) is related with those proposed by Kanazawa *et al.* (18) and Sumida and Tonomura (27). Part II of my thesis deals with work on this line.

I found that two kinds of EP differing in reactivity with ADP are formed in the reaction of SSR ATPase even in the presence of sufficient amounts of KCl, and that ADP-sensitive EP binds 2 mol of Ca^{2+} and ADP-insensitive EP binds 2 mol of Mg^{2+} per mol of EP. ADP-sensitive EP is formed first, then converted into ADP-insensitive EP. The conversion is inhibited by modifying the ATPase with MalNEt. Furthermore, the kinetic properties of the conversion of the two kinds of EP indicated the existence of at least two different conformational states of both ADP-sensitive and -insensitive EP.

REFERENCES

1. Ebashi, S. (1976) *Ann. Rev. Physiol.* 38, 293-313
2. Hasselbach, W., & Makinose, M. (1961) *Biochem. Z.* 333, 518-528
3. Ebashi, S., & Lipmann, F. (1962) *J. Cell Biol.* 14, 389-400
4. Hasselbach, W., & Makinose, M. (1962) *Biochem. Biophys. Res. Commun.* 7, 132-136
5. Hasselbach, W., & Makinose, M. (1963) *Biochem. Z.* 339, 94-111
6. Tada, M., Yamamoto, T., & Tonomura, Y. (1978) *Physiol. Rev.* 58, 1-79
7. Yamamoto, T., Takisawa, H., & Tonomura, Y. (1979) in *Current Topics in Bioenergetics* (Sanadi, R. ed.) Vol 9, Academic Press, New York, in press.
8. MacLennan, D. H. (1970) *J. Biol. Chem.* 245, 4508-4518
9. MacLennan, D. H., Seeman, P., Iles, G. H., & Yip, C. C. (1971) *J. Biol. Chem.* 246, 2702-2710
10. Yamamoto, T., & Tonomura, Y. (1967) *J. Biochem.* 62, 558-575
11. Yamamoto, T., & Tonomura, Y. (1968) *J. Biochem.* 64, 137-145
12. Makinose, M. (1969) *Eur. J. Biochem.* 10, 74-82
13. Martonosi, A. (1967) *Biochem. Biophys. Res. Commun.* 29, 753-757
14. Martonosi, A. (1969) *J. Biol. Chem.* 244, 613-620
15. Inesi, G., & Almendares, J. (1968) *Arch. Biochem. Biophys.* 126, 733-735
16. Inesi, G., Maring, E., Murphy, A. J., & McFarland, B. H. (1970) *Arch. Biochem. Biophys.* 138, 285-294
17. Meissner, G., & Fleischer, S. (1973) *Biochem. Biophys. Res. Commun.* 52, 913-920

18. Kanazawa, T., Yamada, S., Yamamoto, T., & Tonomura, Y. (1971) J. Biochem. 70, 95-123
19. Yamamoto, T., Yoda, A., & Tonomura, Y. (1971) J. Biochem. 69, 807-809
20. Nagano, K., Kanazawa, T., Mizuno, N., Tashima, Y., Nakao, T., & Nakao, M. (1965) Biochem. Biophys. Res. Commun. 19, 759-764
21. Hokin, L. E., Sastry, P. S., Galsworthy, P. R., & Yoda, A. (1965) Proc. Natl. Acad. Sci. U. S. A. 54, 177-184
22. Bastide, F., Meissner, G., Fleischer, S., & Post, R. L. (1973) J. Biol. Chem. 248, 8385-8391
23. Degani, C., & Boyer, P. D. (1973) J. Biol. Chem. 248, 8222-8226
24. Froehlich, J. P., & Taylor, E. W. (1975) J. Biol. Chem. 250, 2013-2021
25. Froehlich, J. P., & Taylor, E. W. (1976) J. Biol. Chem. 251, 2307-2315
26. Tonomura, Y. (1972) Muscle Proteins, Muscle Contraction and Cation Transport, Univ. Tokyo Press & Univ. Park Press, Tokyo & Baltimore
27. Sumida, M., & Tonomura, Y. (1974) J. Biochem. 75, 283-297
28. Shigekawa, M., Dougherty, J. P., & Katz, A. M. (1978) J. Biol. Chem. 253, 1442-1450
29. Shigekawa, M., & Dougherty, J. P. (1978) J. Biol. Chem. 253, 1451-1457
30. Shigekawa, M., & Dougherty, J. P. (1978) J. Biol. Chem. 253, 1458-1464
31. Sumida, M., Kanazawa, T., & Tonomura, Y. (1976) J. Biochem. 79, 259-264
32. Kurzmack, M., & Inesi, G. (1977) FEBS Lett. 74, 35-37

PART I

Factors Affecting the Transient Phase of the
 Ca^{2+} , Mg^{2+} -Dependent ATPase Reaction of
Sarcoplasmic Reticulum from Skeletal Muscle

EXPERIMENTAL PROCEDURE

FSR was prepared from rabbit skeletal muscle as described previously (1). The solubilized ATPase was prepared by the method of le Maire et al. (2). CK [EC 2.7.3.2] was prepared from rabbit skeletal muscle by the method of Noda et al. (3). [γ - 32 P]ATP was synthesized enzymatically by the method of Glynn and Chappell (4). [U- 14 C]ATP was obtained from New England Nuclear Co. (Boston, Mass.) and purified by the method of Cohn and Carter (5).

A Durrum D-133 multi-mixing apparatus was used to follow the rapid reaction. The performance of the instrument was evaluated by following the hydrolysis of 2,4-dinitrophenylacetate with sodium hydroxide at 19°C, as described by Barman and Gutfreund (6). As shown in Fig. 1, the pseudo first-order rate constants at 0.1, 0.2 and 0.3 M NaOH were 3.7, 7.0 and 11.7 s⁻¹, respectively. The second-order rate constant, 37 M⁻¹ s⁻¹, was consistent with the value previously obtained by Barman and Gutfreund (6), using a stopped-flow method. The ATPase reaction of FSR was started by mixing the enzyme solution in syringe A with the substrate solution in syringe B. At intervals, the reaction was quenched with 1 N HCl from syringe C. The mixing ratio of syringes A:B:C was 1:1:2. The protein concentration in the enzyme solution was 0.3 mg/ml and the ATP concentration in the substrate solution was 20 μ M. Both the enzyme and substrate solutions contained 0.1 M KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mM EGTA, and 20 mM Tris-maleate at pH 7.0 and 20°C, unless otherwise stated.

The amount of EP and the ATPase activity in the steady state were measured on the solubilized SR. The reaction was started by

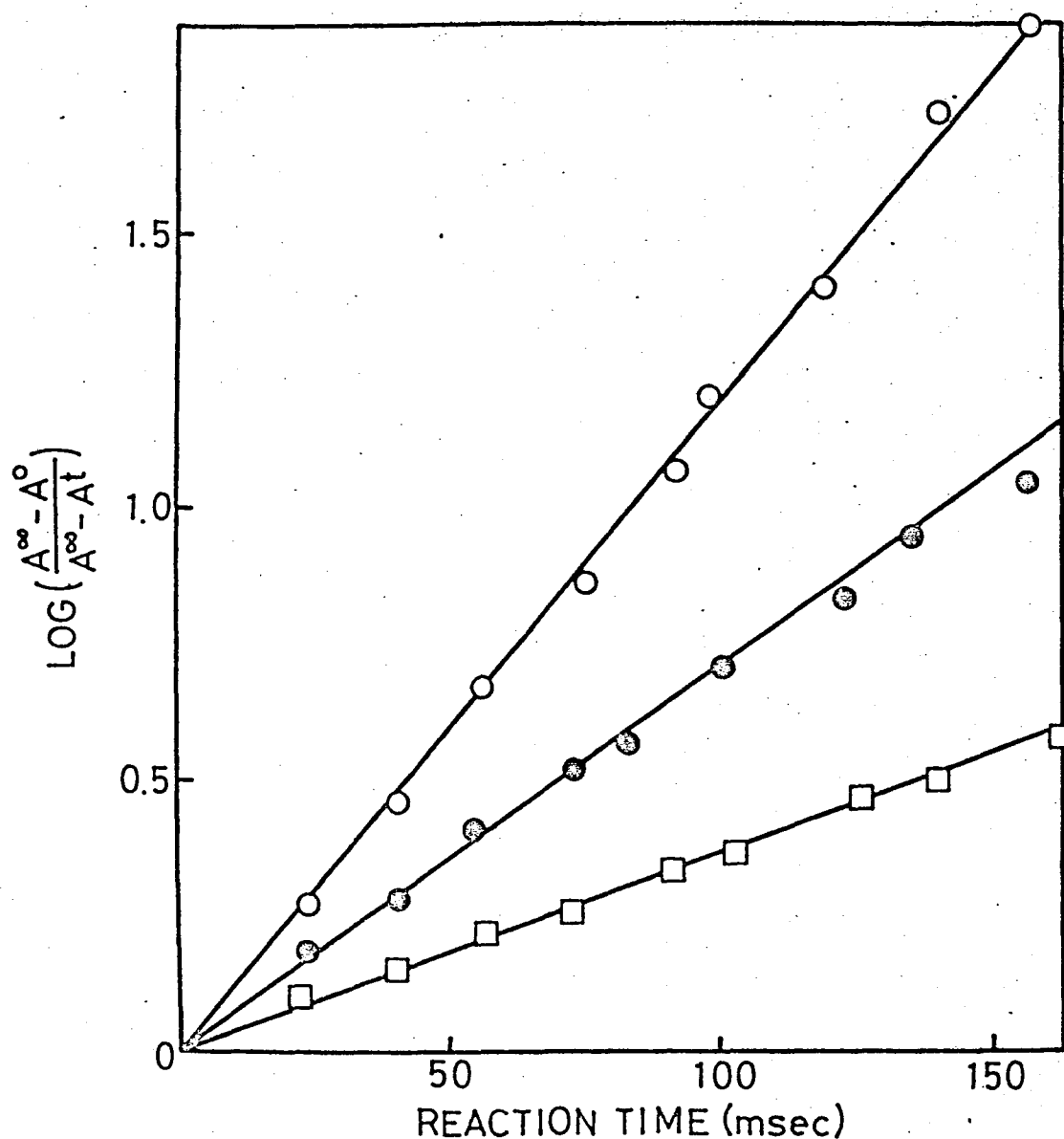


Fig. 1. First-order plot of alkaline hydrolysis of 2,4-dinitrophenyl acetate measured using a multi-mixing apparatus. 2,4-Dinitrophenyl acetate (0.5 mM) was hydrolyzed in 0.1 (□), 0.2 (◐), and 0.3 M (O) NaOH at 19°C, respectively. The absorbance of 2,4-dinitrophenol liberated was measured at 360 nm. A_{∞} represents the maximum absorbance change for the reaction. A_t and A_0 represent the absorbance at time t and that at time 0 after the start of the reaction, respectively.

adding ATP to the reaction mixture to give the final concentrations of 0.1 M KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mg/ml Tween 80, and 20 mM Tris-maleate at pH 7.0 and 20°C. The reaction was stopped by adding 6% TCA.

Next, a 1-ml portion of the quenched suspension was added to 1 ml of 4% TCA containing 0.1 mM unlabeled ATP and 0.2 mM P_i as carriers. The suspension was centrifuged at 1000 x g for 20 min, then ³²P_i in the supernatant was extracted by the method of Martin and Doty (7) and its radioactivity was measured as described previously (8). The denatured SR protein was washed on a Millipore filter and the amount of E³²P was measured as described previously (9).

The amount of ADP bound to the solubilized enzyme during the ATPase reaction was determined by measuring the amount of ADP remaining in the SR ATPase-ATP system coupled with sufficient amounts of CK and CP (10). The reaction was started by adding [¹⁴C]ATP to the reaction mixture to give the final concentrations of 0.055 mg/ml SR protein, 5 mg/ml CK, 0.5 μM ATP, 10 mM CP, 50 mM KCl, 50 μM CaCl₂, 10 mM MgCl₂, 0.05 mg/ml Tween 80, and 0.1 M Tris-HCl at pH 9.0 and 20°C. After the reaction had been stopped, the amounts of nucleotides were measured using polyethyleneimine cellulose TLC, as described previously (10). The amount of EP was measured under the same conditions, except that AT³²P was used instead of [¹⁴C]ATP and CP was omitted.

Protein concentration was determined by the biuret reaction, calibrated by nitrogen determination.

RESULTS

Time Courses of P_i Liberation and EP Formation of FSR in the Presteady State — As discussed in the "INTRODUCTION," the ATPase reaction of the FSR preparation showed an initial burst of P_i liberation even in the absence of Ca^{2+} ions. This complicated the analysis of the Ca^{2+} , Mg^{2+} -dependent ATPase reaction during the initial phase. I found that the size of the P_i burst of the ATPase reaction in the absence of Ca^{2+} ions varied among FSR preparations. Therefore, to avoid complication due to the P_i burst of the Ca^{2+} -independent ATPase reaction, I performed the following experiments using FSR preparations which showed low activity in the absence of Ca^{2+} ions, and also using low ATP concentrations where the Ca^{2+} -independent ATPase activity was low.

Figure 2 shows the time course of P_i liberation and EP formation in the initial phase of the Ca^{2+} , Mg^{2+} -dependent ATPase reaction. The reaction was performed under the standard conditions, except that the enzyme solution contained 0.2 mg/ml SR protein. The amount of EP increased rapidly after the reaction had been started by adding ATP, then decreased slightly after reaching a maximum value (6 $\mu\text{mol/g}$ protein) at 50 ms. Then 0.1 s after the reaction had been started, the amount of EP began to increase gradually and reached a steady-state level (about 6 $\mu\text{mol/g}$ protein) at 1 s. The amount of P_i liberated increased rapidly after a short lag, and the rate of P_i liberation decreased markedly with time during the first 1 s of the reaction. During the first 1 s, 24 $\mu\text{mol/g}$ protein of P_i was liberated, whereas the amount liberated 1 to 2 s after the start of the reaction was only 5 $\mu\text{mol/g}$ protein.

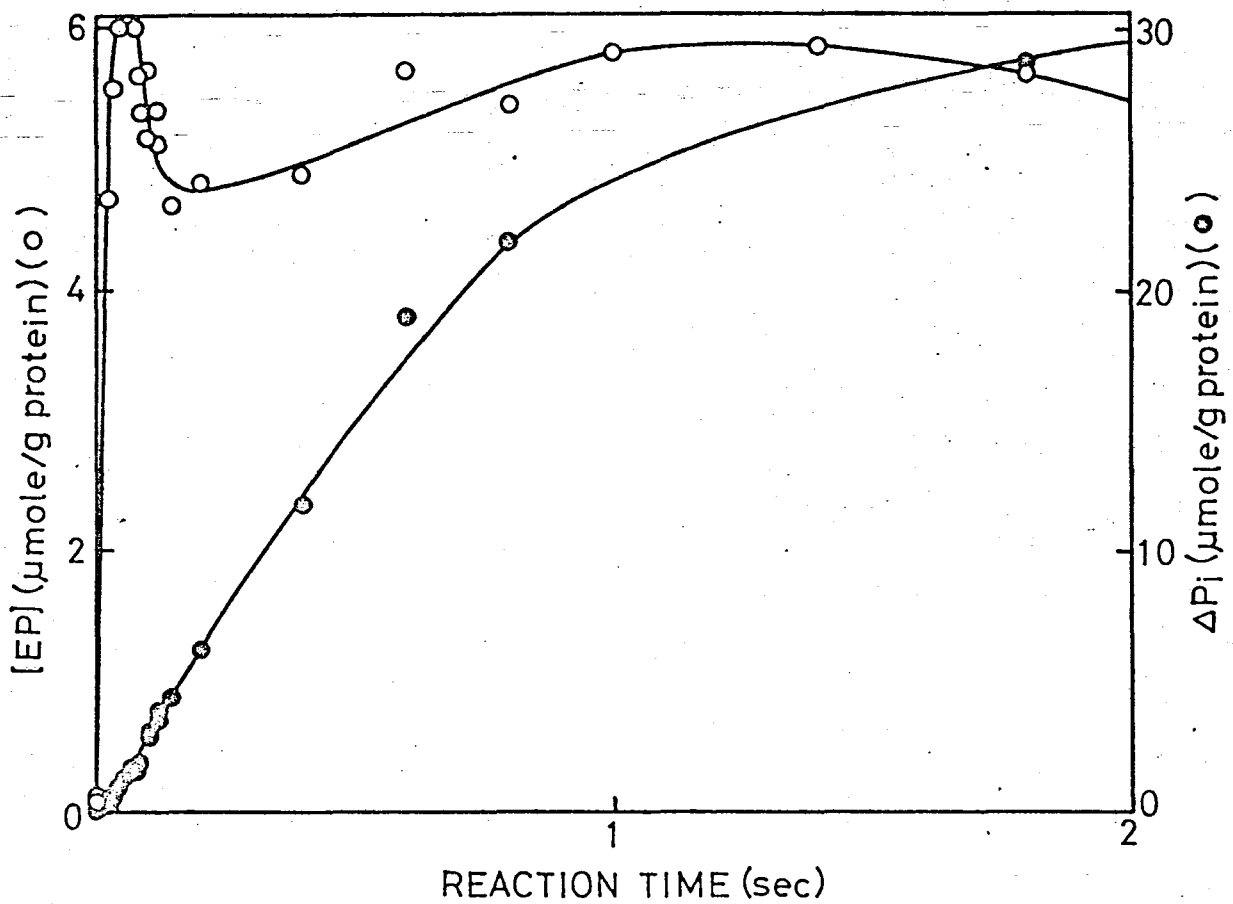


Fig. 2. Time courses of EP formation and P_i liberation during the initial two seconds of the reaction. Both the enzyme and the substrate solutions contained 0.1 M KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM EGTA, and 20 mM Tris-maleate at pH 7.0 and 20°C. The final concentrations of FSR protein and ATP were 0.1 mg/ml and 10 μ M, respectively. The reaction was performed as described in the "EXPERIMENTAL PROCEDURE." The amounts of P_i liberated (●) and EP formed (○) were measured.

Comparison of the Time Courses in the Presteady State of Reactions Started by Addition of ATP and ATP + Ca^{2+} — Figure 3 shows the differences between the initial phases of ATPase reactions started by addition of ATP and ATP + Ca^{2+} . The former reaction was performed under the standard conditions, the latter under conditions under which the enzyme solution contained 0.2 mM EGTA instead of 0.1 mM EGTA + 0.1 mM CaCl_2 and the final reagent concentrations were the same as those of the former. When the reaction was started by ATP, the EP overshoot was observed: the amounts of EP were 3.2 and 2.8 $\mu\text{mol/g}$ protein, respectively, at the maximum level and in the steady state. The time course of P_i liberation showed a lag phase of about 20-30 ms, and the amount of P_i liberated was very small even when the amount of EP approached the maximum value. After the lag phase, the P_i burst was observed, and its time course coincided with that of the transient decay in EP. On the other hand, when the reaction was started by addition of Ca^{2+} + ATP, the EP overshoot was not observed, and the amount of P_i increased almost linearly with time after a lag phase. The amount of EP in the steady state was 2.2 $\mu\text{mol/g}$ protein, which was slightly less than that of the ATP start (2.8 $\mu\text{mol/g}$ protein). The steady-state rate of P_i liberation with the Ca^{2+} + ATP start was 21 $\mu\text{mol}/(\text{g protein}\cdot\text{s})$, which was slightly lower than that with the ATP start [25 $\mu\text{mol}/(\text{g protein}\cdot\text{s})$].

Effect of Preloading FSR with Ca^{2+} Ions on the Presteady State Reaction — FSR accumulates Ca^{2+} ions inside the vesicle coupled with ATP hydrolysis, and accumulated Ca^{2+} ions leak out gradually, reaching equilibrium in a few hours after complete hydrolysis of ATP (cf. ref. 11 and 12). FSR was loaded with Ca^{2+} by preincubating

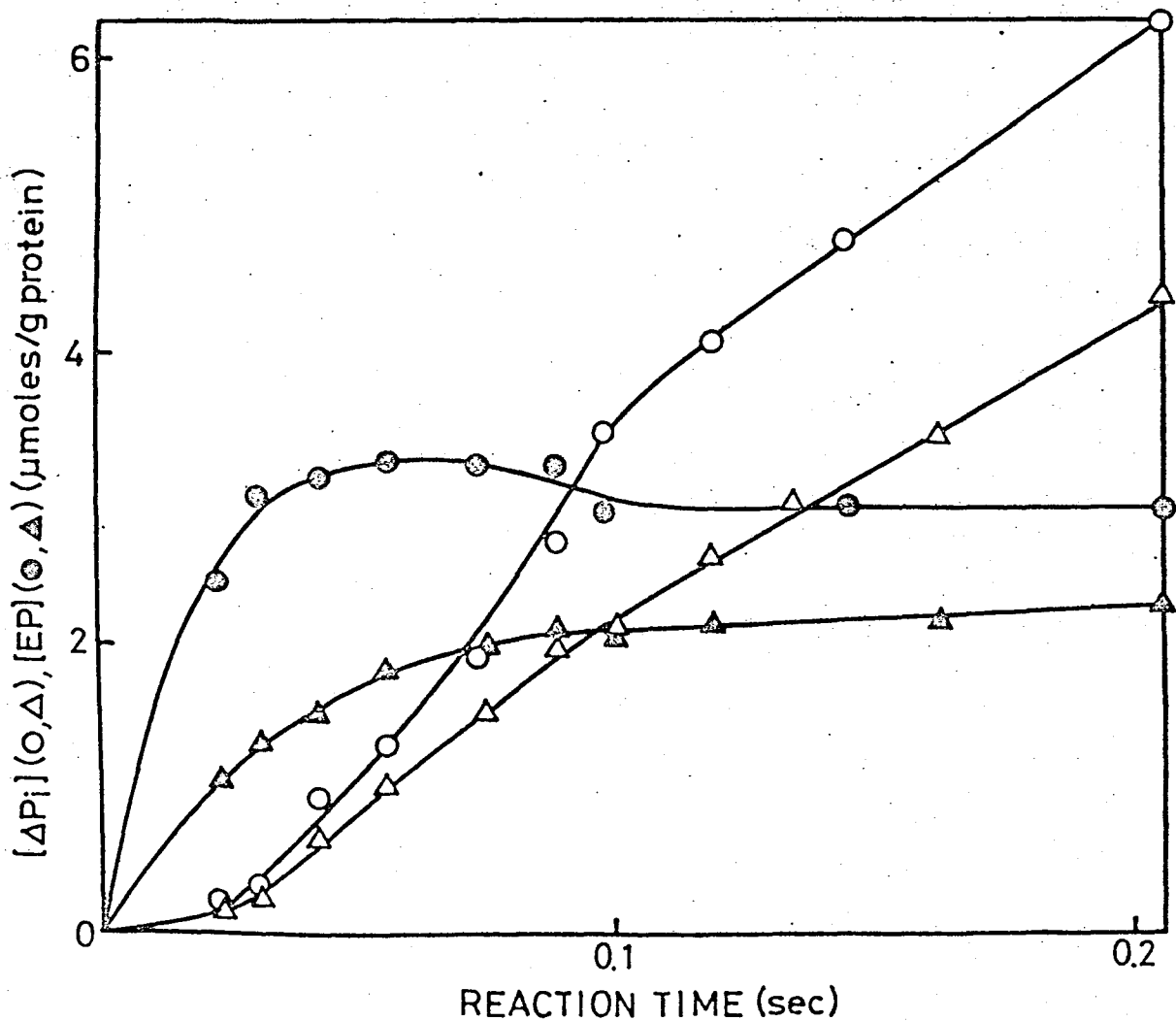


Fig. 3. Absence of the EP overshoot and the P_i burst after starting the reaction by addition of ATP together with Ca^{2+} ions. The reaction started by addition of ATP (\circ, \bullet) was performed under the standard conditions. The reaction started by addition of ATP + Ca^{2+} ($\triangle, \blacktriangle$) was performed under the standard conditions, except that the enzyme solution contained 0.2 mM EGTA instead of 0.1 mM CaCl_2 and 0.1 mM EGTA. The amounts of P_i liberated (\circ, \triangle) and EP formed (\bullet, \blacktriangle) were measured.

the enzyme solution (0.3 mg/ml SR protein) with 10 μM unlabeled ATP in 1 mM MgCl_2 , 0.1 mM CaCl_2 , and 0.1 mM EGTA. After 1-min preincubation, the reaction was started by adding AT^{32}P under the standard conditions. A control reaction was performed under the same conditions, except that the enzyme solution was preincubated with 10 μM ADP instead of ATP. Figure 4 shows that in the control experiment, the EP overshoot was observed and the maximal amount of EP was 2.4 $\mu\text{mol/g}$ protein, while the amount in the steady state was 2.0 $\mu\text{mol/g}$ protein. The time course of P_i liberation showed a lag phase, and the amount of P_i liberated was very small, even when the amount of EP approached the maximum value. After the lag phase, a typical P_i burst was also observed (cf. Fig. 3). On the other hand, when the FSR was preloaded with Ca^{2+} , the EP overshoot and the P_i burst did not occur. The rate of P_i liberation in the steady state was 12.5 $\mu\text{mol}/(\text{g protein}\cdot\text{s})$, which was slightly lower than that of the control [18.2 $\mu\text{mol P}_i/(\text{g protein}\cdot\text{s})$]. The amount of EP in the steady state was 3 $\mu\text{mol/g}$ protein, which was larger than that of the control. Thus, the apparent rate constant of EP decomposition, $v/[\text{EP}]$, decreased from 9.1 to 6.1 s^{-1} with the Ca^{2+} preloading. The decrease in $v/[\text{EP}]$ may be due to the inhibition of EP decomposition by Ca^{2+} accumulated inside the vesicle (cf. ref. 11).

To confirm that these changes in the ATPase reaction were caused by Ca^{2+} inside the vesicle, the reaction was started by adding AT^{32}P after FSR had been preincubated with unlabeled ATP for various time intervals (Fig. 5). When the reaction was started by adding AT^{32}P after 10-min preincubation with unlabeled ATP, neither the EP overshoot nor the P_i burst were observed. However,

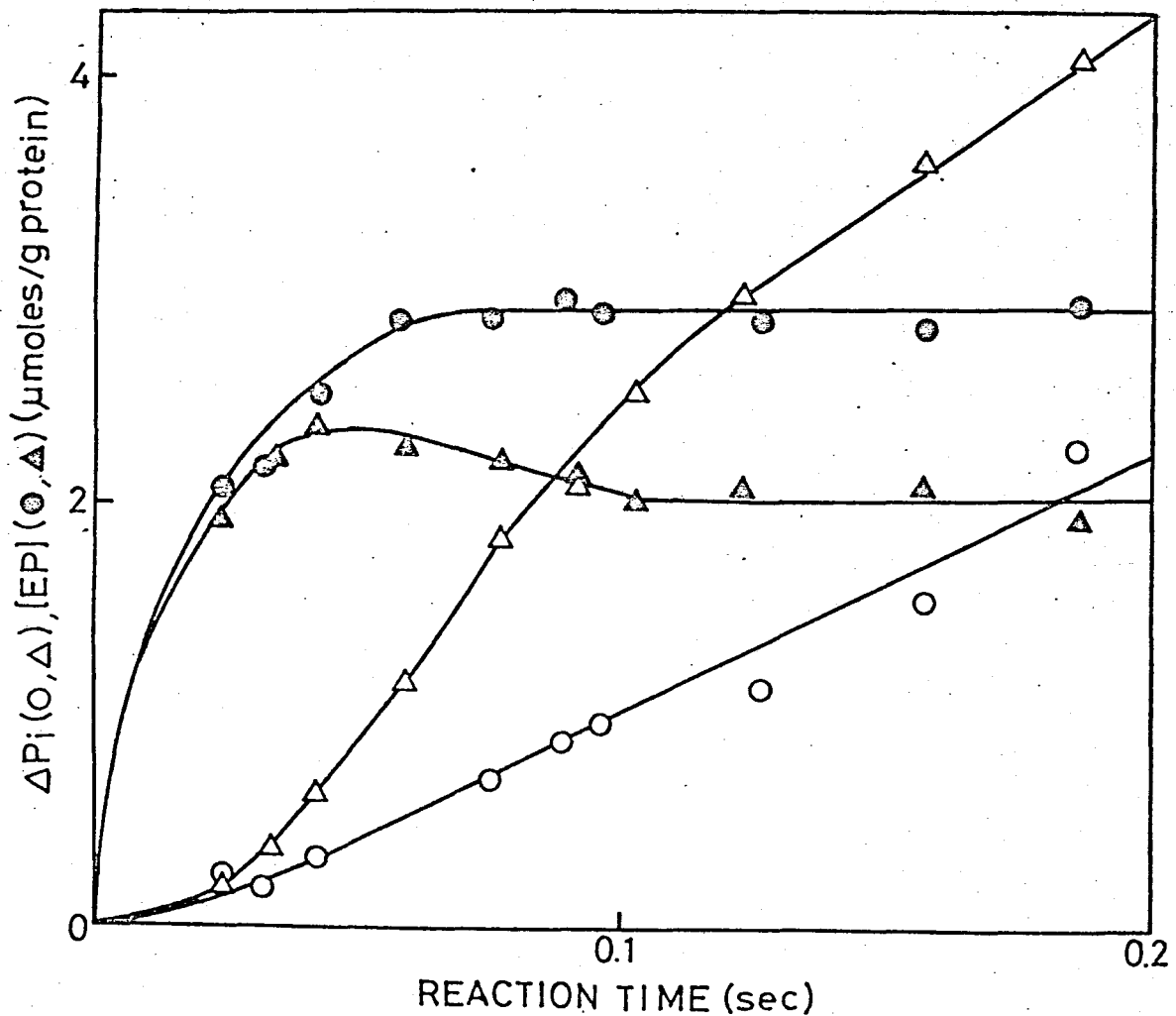


Fig. 4. Effect of preloading of Ca^{2+} ions on the presteady state of the ATPase reaction. FSR (0.3 mg/ml SR protein) was suspended in 0.1 M KCl, 1 mM MgCl_2 , 0.1 mM CaCl_2 , 0.1 mM EGTA and 20 mM Tris-maleate at pH 7, and preincubated with 10 μM unlabeled ATP (○,●) or ADP (Δ,▲) at 20°C for 1 min. After preincubation, the reaction was started by adding 10 μM [γ - ^{32}P]ATP to FSR under the standard conditions. The amount of $^{32}\text{P}_i$ liberated (○,Δ) and E^{32}P formed (●,▲) were measured.

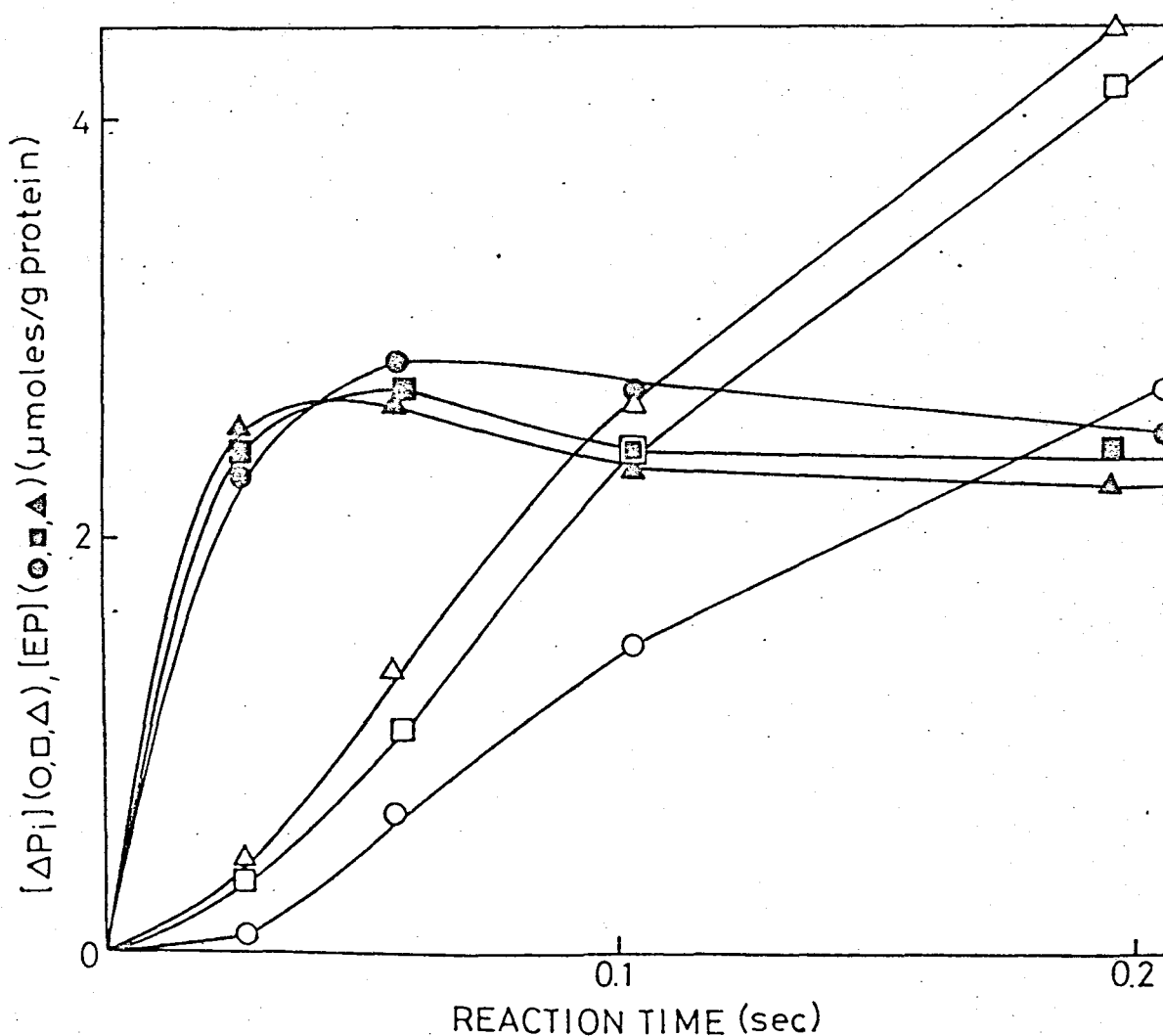


Fig. 5. Appearance of the EP overshoot and the P_i burst of the first type after long preincubation of Ca^{2+} -preloaded FSR with EGTA. The enzyme solution was preincubated with 10 μM unlabeled ATP as described in Fig. 4. The preincubation times were 10 (○, ●), 100 (□, ■) and 200 min (Δ, ▲), respectively. After a long preincubation, the unlabeled ATP added was hydrolyzed completely and Ca^{2+} ions preloaded into FSR had almost completely leaked out, as the outside solution contained 0.1 mM EGTA. The reaction was started by adding 10 μM $AT^{32}P$ to FSR, and the amounts of $^{32}P_i$ liberated (○, □, Δ) and $E^{32}P$ formed (●, ■, ▲) were measured under the standard conditions.

when the preincubation time was longer than 100 min, they occurred. The reaction profile of the ATPase after 200-min preincubation with unlabeled ATP was very similar to that of the control shown in Fig. 3. According to Yamada et al. (13), after 100-200 min incubation of FSR, the concentration of Ca^{2+} ions reaches an equilibrium across the FSR membrane.

Time Course of P_i Liberation and EP Formation of FSR ATPase at 4°C — To clarify the relationship between the rate of P_i liberation and the amount of EP in the initial phase of the reaction, I performed the ATPase reaction under the standard conditions at a low temperature, i.e., 4°C, to lower the ATPase activity. As shown in Fig. 6, the amount of EP increased with time without a lag phase and reached a maximum value (5.4 $\mu\text{mol/g}$ protein) 0.4 s after the reaction had been started. The time course of P_i liberation consisted of a long lag phase, a burst phase and a steady phase. Figure 7 shows the change in the apparent rate constant of EP decomposition ($k_d = v/[\text{EP}]$) with time after the start of the reaction. The value of v at time t was calculated as $\Delta \text{P}_i / \Delta t$, where ΔP_i is the amount of P_i liberated during Δt around the time t . The k_d value was 0.5 s^{-1} in the initial phase, increased to a maximum of 1.8 s^{-1} after 1.3 s of reaction, then decreased rapidly to the steady value (0.1 s^{-1}) 2 s after the reaction start. Changes in k_d with time similar to those observed at 4°C also occurred at 20°C, although the k_d value during this initial phase could not be determined accurately.

Time Course of P_i Liberation and EP Formation of Solubilized ATPase in the Presteady State — To investigate whether the EP overshoot and the P_i burst in the initial phase of the FSR ATPase

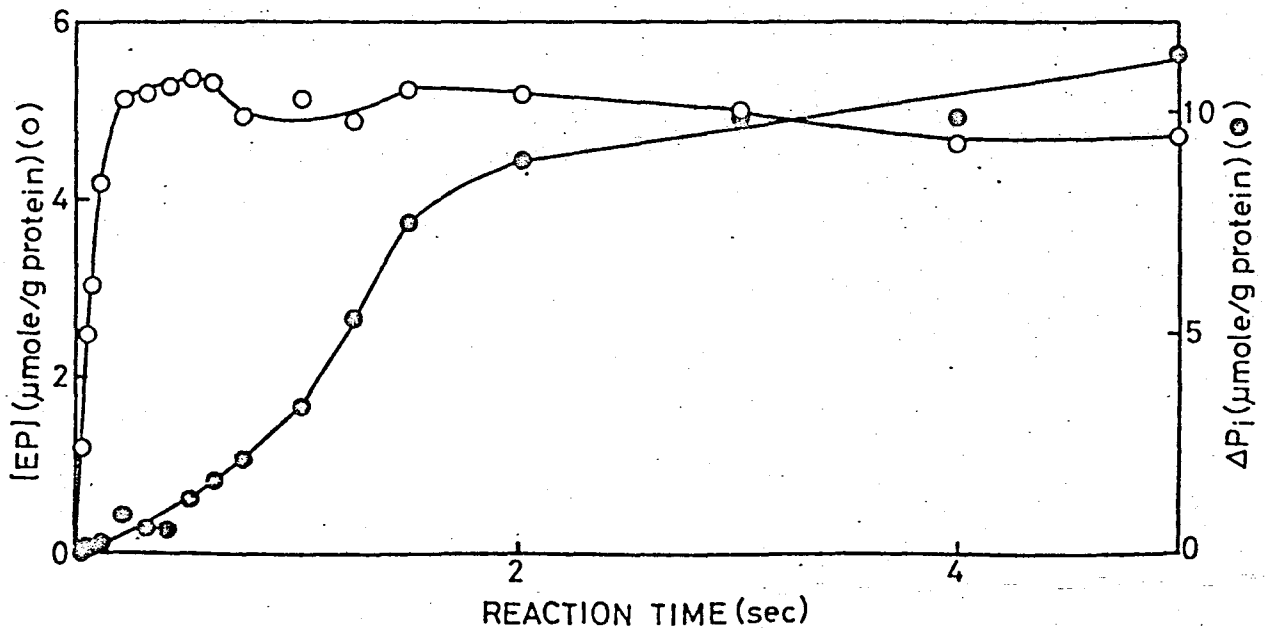


Fig. 6. Time courses of EP formation and P_i liberation during the initial phase at 4°C . The time courses of P_i liberation (\bullet) and EP formation (\circ) were measured under the standard conditions, except that the SR protein concentration was 0.14 mg/ml and the temperature was 4°C instead of 20°C .

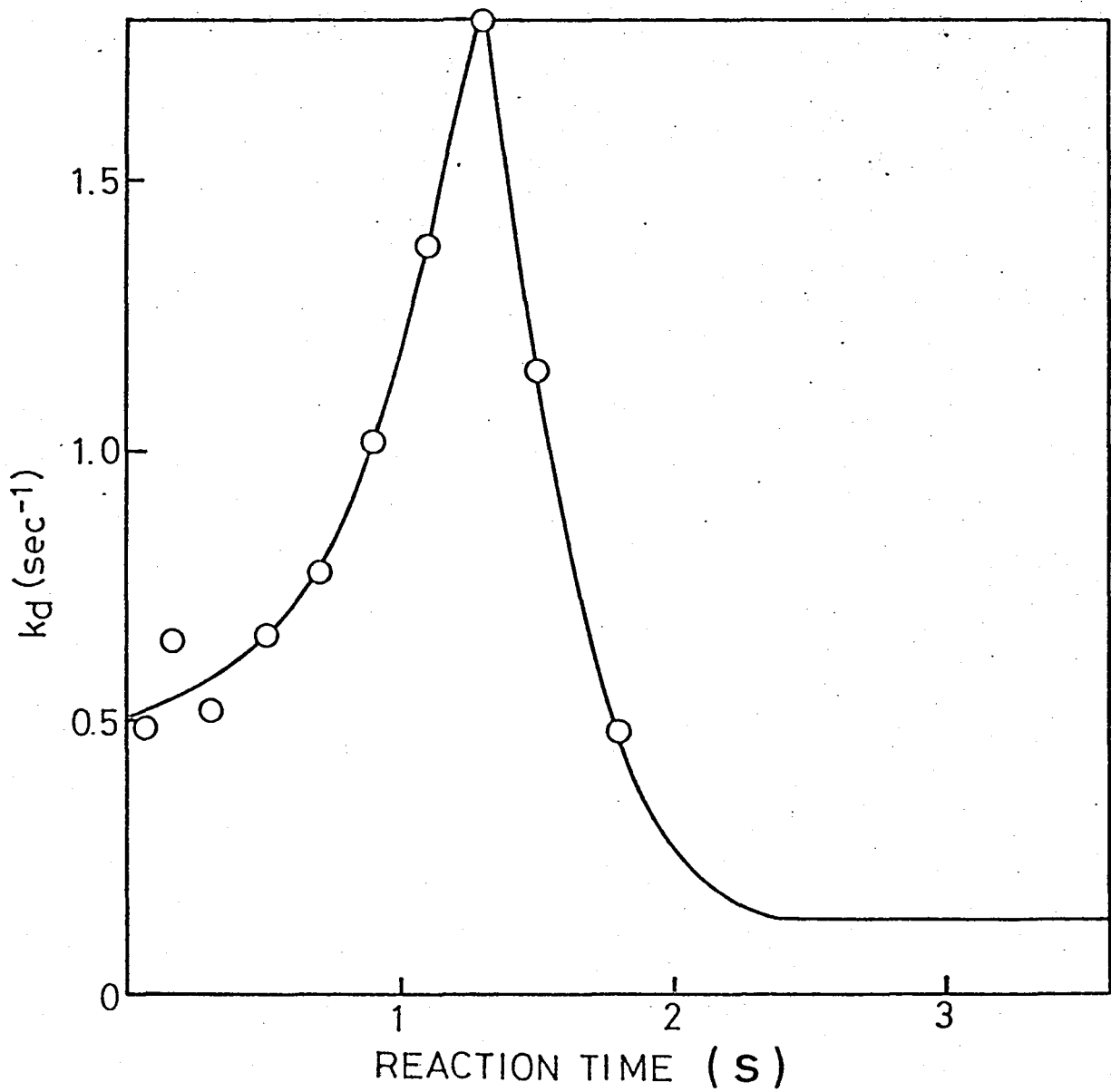


Fig. 7. Time course of change in the k_d value during the initial phase at 4°C. The k_d value was obtained by dividing v by $[EP]$ at time t given in Fig. 6. The value of v at time t was estimated as $\Delta P_i / \Delta t$ as described in the text.

reaction are related to the membrane structure, the reaction of the ATPase solubilized by the method of le Maire et al. (2) was studied in 0.1 M KCl, 1 mM MgCl_2 , and 0.1 mM CaCl_2 in the presence of 0.1 mg/ml of Tween 80 at pH 7.0 and 20°C. Figure 8 shows the time course of the EP formation and P_i liberation in the presteady state after 10 μM ATP had been added to 0.125 mg/ml SR. The amount of EP increased rapidly with time and reached the steady-state level, 2.83 $\mu\text{mol/g}$ protein, within 0.06 s; the EP overshoot was not observed. The P_i liberation showed a definite presteady state which closely corresponded to the period when the amount of EP was increasing. The observed time course of P_i liberation agreed well with that calculated from the observed time course of EP formation on the assumption that EP was an intermediate in the reaction and its specific turnover rate was 7.1 s^{-1} .

Dependence on ATP Concentration of the Rate of P_i Liberation and the Amount of EP of Solubilized ATPase in the Steady State — The dependence on the ATP concentration of the ATPase activity and the amount of EP of the solubilized ATPase in the steady state was measured under the standard conditions (Fig. 9). The double reciprocal plots of the ATPase activity and the amount of EP against the ATP concentration gave straight lines, and the Michaelis constants both for the ATPase activity and the EP formation were 1 μM , which were almost equal to the values obtained on FSR (8). The maximum rate of P_i liberation was 14.5 $\mu\text{mol P}_i/(\text{g protein} \cdot \text{s})$, which was much higher than the 2.2 $\mu\text{mol P}_i/(\text{g protein} \cdot \text{s})$ obtained on FSR (8). On the other hand, the maximum amount of EP was 3 $\mu\text{mol/g}$ protein, which was smaller than the 6 $\mu\text{mol/g}$ protein of FSR (8). Therefore, the value of $v/[\text{EP}]$ of

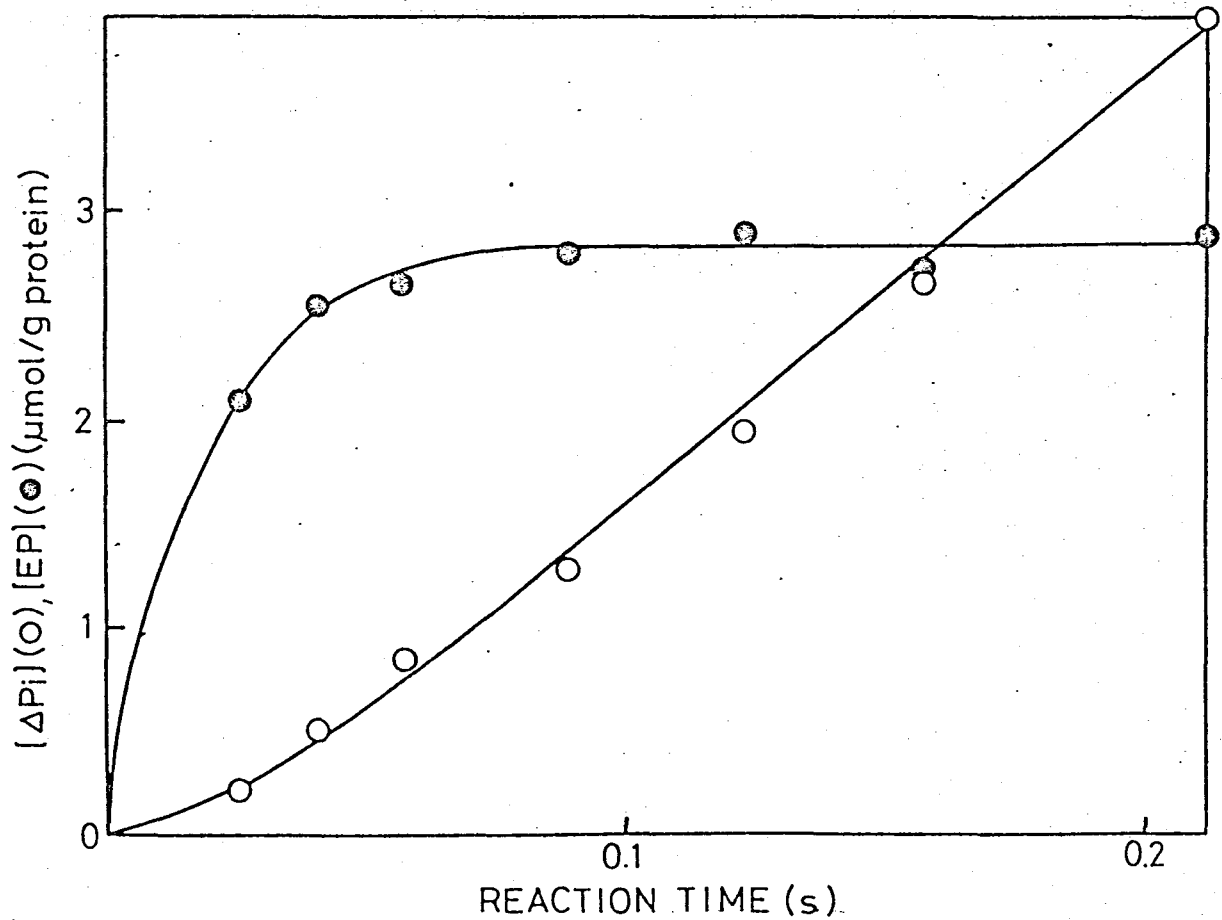


Fig. 8. Time courses of EP formation and P_i liberation of the solubilized ATPase during the initial phase of the reaction. The ATPase reaction of the solubilized SR was performed in 0.1 M KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1 mg/ml Tween 80, and 20 mM Tris-maleate at pH 7.0 and 20°C. The reaction was started by adding 10 μ M ATP to 0.125 mg/ml SR, and the amounts of P_i liberated (O) and EP formed (●) were measured. The line for P_i liberation was calculated by assuming that the rate constant for EP decomposition was 7.1 s^{-1} .

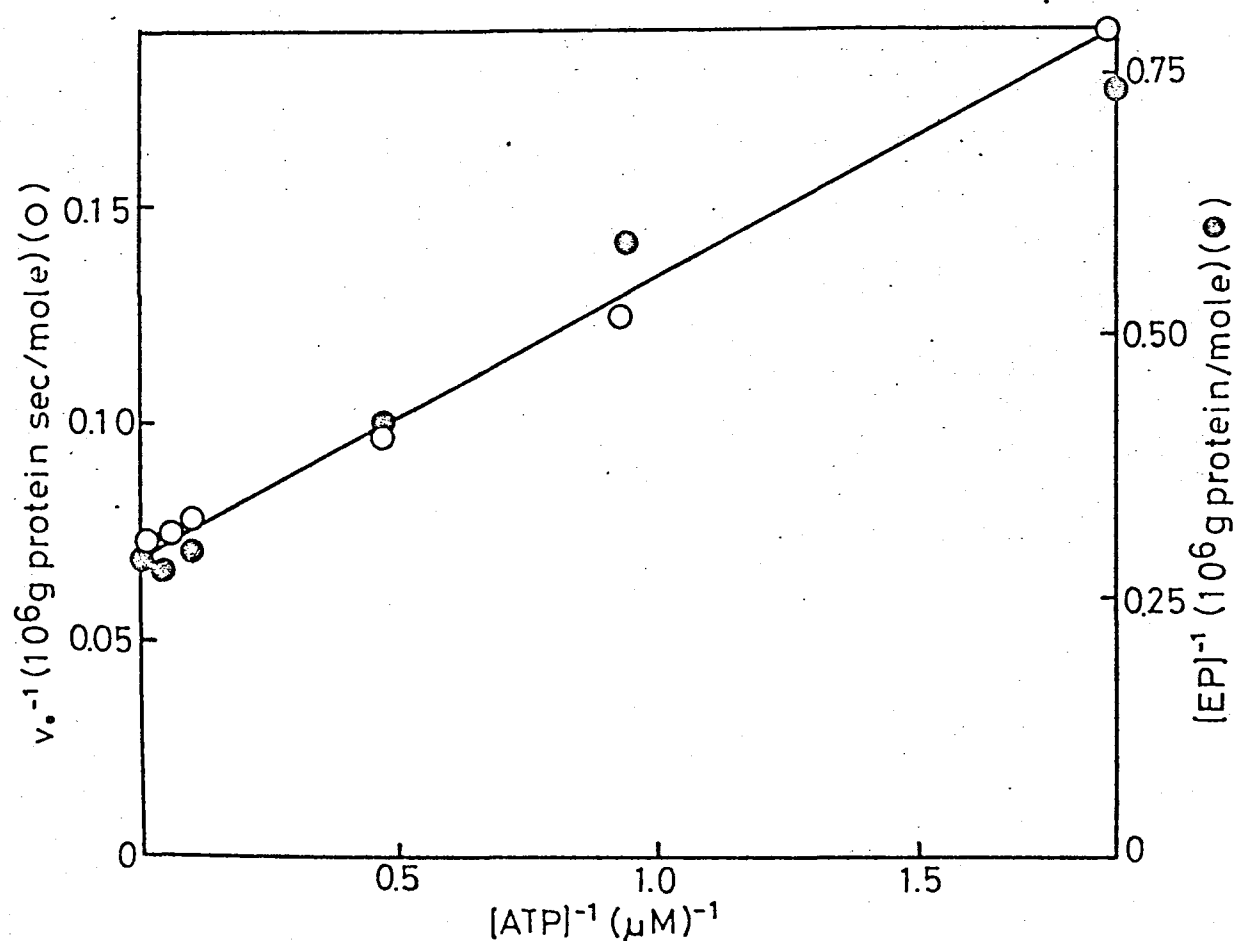


Fig. 9. Double reciprocal plots of the rate of the ATPase reaction and the amount of EP of the solubilized ATPase in the steady state against the ATP concentration. The rate of ATPase (O) and the amount of EP (\bullet) were measured in the presence of 7 (O) or 40 (\bullet) $\mu\text{g/ml}$ SR protein in 0.1 M KCl, 1 mM MgCl_2 , 0.1 mM CaCl_2 , 0.1 mg/ml Tween 80, and 10 mM Tris-maleate at pH 7.0 and 20°C.

solubilized ATPase (4.2 s^{-1}) was about ten times larger than that of FSR. Acceleration of the ATPase activity by dissolving FSR has already been reported by many investigators (14,15).

ADP Binding to SR ATPase during the ATPase Reaction — I measured the amount of ADP bound to the solubilized ATPase during the ATPase reaction by the method described in the "EXPERIMENTAL PROCEDURE." Figure 10 shows the time courses of increase in the amount of EP and that of ADP remaining after starting the reaction with ATP under the standard conditions. The amount of EP in the steady state was $1.8 \text{ } \mu\text{mol/g}$ protein, which was equivalent to the concentration of $0.1 \text{ } \mu\text{M}$ in the experimental conditions used, as shown in the figure. The [^{14}C]ATP preparation used in this experiment was contaminated by a trace amount of AMP, and the amount of AMP increased slightly during the ATPase reaction, probably due to the presence of membrane-bound adenylate kinase activity [EC 2.7.4.3]¹. No ADP was observed in this reaction system, and the amount of ATP remained at $0.48 \text{ } \mu\text{M}$. I thus concluded that the main reaction intermediates in the steady state do not contain bound ADP.

DISCUSSION

The EP overshoot reported by Froehlich and Taylor (16) was observed at about 50 ms after the start of the reaction, and the P_i liberation also showed a burst phase which coincided with the transient decay of EP (see Figs. 2 & 3). However, the transition of the k_d value ($v/[\text{EP}]$) continued for longer than 1 s after the EP overshoot had been completed, and the burst size of P_i liberation due to

¹Watanabe, K., Itakura, K., and Kubo, S., personal communication

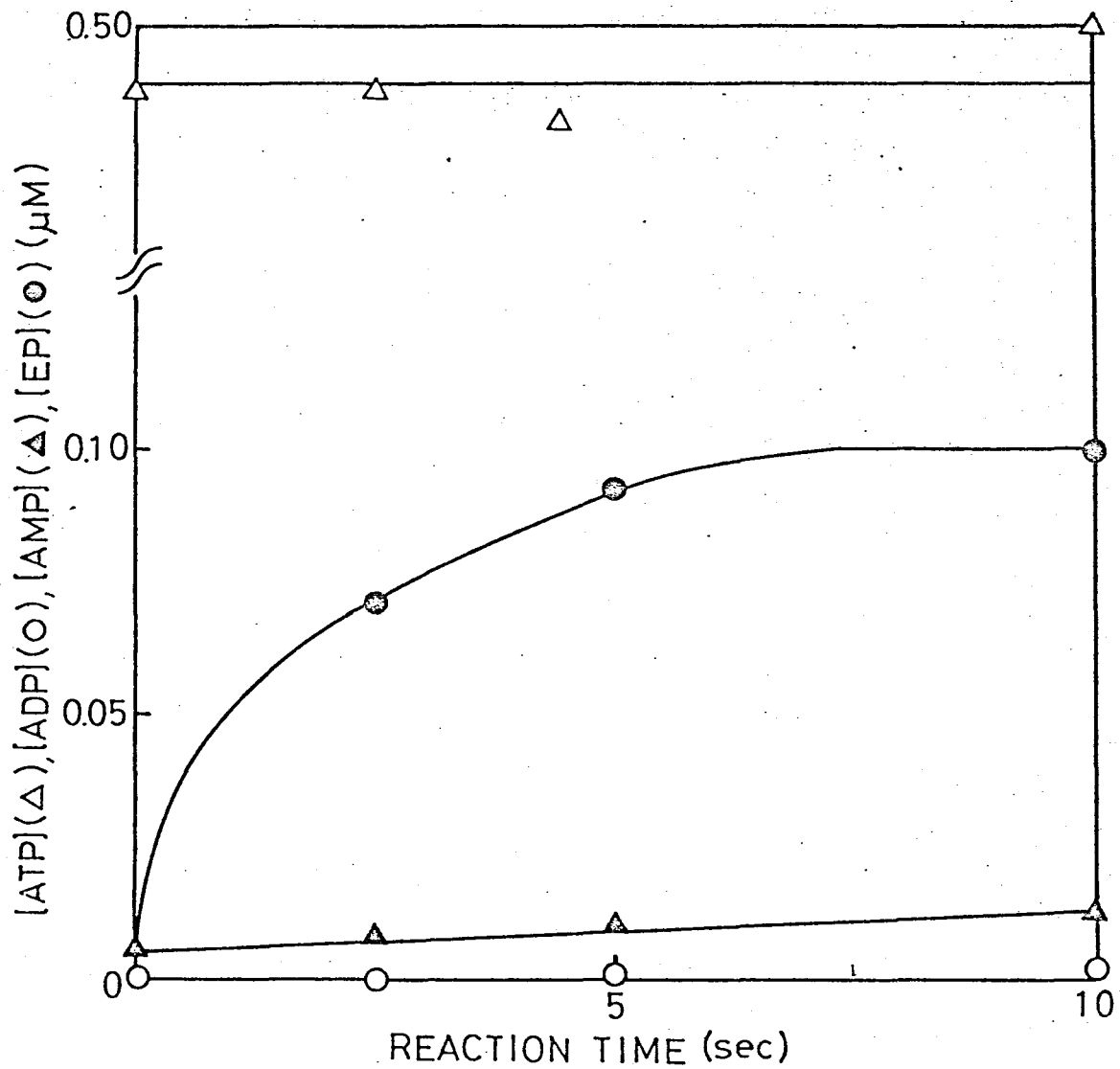


Fig. 10. Evidence for lack of ADP binding to EP. The amounts of ATP (Δ), ADP (\circ) and AMP (\blacktriangle) were measured in 55 $\mu\text{g/ml}$ SR protein, 0.5 μM ATP, 50 mM KCl, 50 μM CaCl_2 , 10 mM MgCl_2 , 0.05 mg/ml Tween 80, and 0.1 M Tris-HCl at pH 9.0 and 20°C. The ATPase reaction was coupled with 5 mg/ml CK and 10 mM CP to convert free ADP completely to ATP. The amount of EP (\odot) was measured in the absence of CP.

the $v/[EP]$ transition was about 20 $\mu\text{mol/g}$ protein (Fig. 2). Since the time range of the P_i burst related to the transient decay of EP and that of the k_d transition were different, and since the burst size of P_i liberation due to the k_d transition was much larger than the active site concentration, it is reasonable to assume that the reaction mechanisms for these two kinds of P_i burst are different. Hereafter, I will call the P_i burst observed by Froehlich and Taylor (16) the P_i burst of the first type, and that observed by Kanazawa et al. (8) the P_i burst of the second type.

Froehlich and Taylor (16) asserted that the P_i burst of the first type is caused by the formation of an acid-labile intermediate ($E \cdot P$) formed by rapid hydrolysis of EP. Although the possibility of an appreciable amount of $E \cdot P$ existing in equilibrium with EP in the steady state could be excluded, the possibility of $E \cdot P$ existing in the transient phase could not (9). However, Boyer et al. (17) conducted kinetic analyses of the reverse reaction, that is, the $P_i \rightleftharpoons \text{HOH}$ exchange reaction and the EP formation from medium P_i , and showed that the Michaelis complex ($E \cdot P_i$) is in rapid equilibrium with medium P_i (the rate constant of decomposition of $E \cdot P_i$ into $E + P_i$ calculated by me from Boyers' results was higher than 200 s^{-1} , while the value assumed by Froehlich and Taylor (16) was 10 s^{-1}). Thus, the $E \cdot P$ of Froehlich and Taylor can not be considered the Michaelis complex ($E \cdot P_i$) formed in the reverse reaction. Furthermore, the complicated kinetic behavior of the initial phase could not be explained by the mechanism of Froehlich and Taylor. When the reaction was started by adding $\text{Ca}^{2+} + \text{ATP}$, neither the EP overshoot nor the P_i burst of the

first type occurred (Fig. 3), although both the rate of P_i liberation and the amount of EP in the steady state were almost equal to those obtained when the reaction was started with ATP. These great differences between the reaction profile in the initial phase of the ATP start and that of the $Ca^{2+} + ATP$ start can not be due to a slow binding of Ca^{2+} ions to the ATPase, because Ca^{2+} ions bind very rapidly with the SR ATPase (18) and Ca^{2+} and ATP bind with the ATPase in a random sequence (8). The EP overshoot and the P_i burst of the first type were also eliminated when SR was preloaded with a small amount of Ca^{2+} ions (Figs. 4 and 5), although the rate of P_i liberation and the amount of EP in the steady state were affected only slightly.

The complicated relationship between the rate of P_i liberation and the amount of EP in the presteady state was clearly evident when the ATPase reaction was performed at 4°C. The plot of the "apparent" rate constant of EP decomposition ($v/[EP]$) versus time showed a bell-shaped profile (Fig. 7). Neither the mechanism proposed by Kanazawa et al. (8) nor that by Froehlich and Taylor (16) can explain this complicated time course of the $v/[EP]$ value. The long lag phase of P_i liberation may be explained if I assume that the acid-labile P_i is formed through at least two TCA-stable EP intermediates. Ikemoto (19) also proposed the sequential formation of two acid-stable EP intermediates differing in Ca^{2+} affinity.

Since the existence of an acid-labile intermediate ($E \cdot P$) is doubtful under my experimental conditions, I must consider other possibilities to explain the kinetics of the ATPase reaction in the initial phase. One way is to propose a complicated reaction

scheme with an increased number of intermediates. For example, the possibility exists that P_i is liberated not only from the EP intermediate but also from other intermediates such as EATP, as suggested by Lowe *et al.* (20) based on analysis of the Na^+ , K^+ -dependent ATPase [EC 3.1.6.3] reaction in the presteady state. Another possibility is that two EP intermediates exist and they have different rate constants for decomposition, which was proposed by Nakamura and Tonomura (21) to explain the presteady-state and steady-state kinetic behaviors of the SR p-nitrophenylphosphatase reaction. A second way is to assume that "apparent" rate constants of several steps change with the reaction time. Sumida *et al.* (9) have already shown that the P_i burst of the second type is due to the change of the "apparent" rate constant of EP decomposition with time. A third way is to assume interaction between ATPase molecules. For example, Fig. 11 shows an attempt to explain the bell-shaped profile of the time course of $v/[EP]$, on the assumption that two EP intermediates interact with each other and the rate constant of decomposition of one EP is proportional to the amount of decomposition of the other EP.

In the above analyses, I tacitly assumed that the physico-chemical state of ATPase is homogeneous at the start of the reaction. However, Yamamoto and Tonomura (22) showed that the number of lysine residues in various subfragments of SR ATPase, which are modified by 2,4,6-trinitrobenzenesulfonate, is not integral. Therefore, they suggested the possibility that the state of the ATPase in the membrane is somewhat heterogeneous (22). Thus, it is essential to know whether the interactions between the ATPase molecule and PL are the same for all the ATPase molecules in the

membrane. When the ATPase reaction was performed on solubilized ATPase in the presence of Tween 80, both the EP overshoot and the P_i burst of the first type did not occur (Fig. 8), and the lag phase in the P_i liberation corresponded closely to the period when the EP concentration was increasing, as already reported by Yamada et al. (15) using Triton X-100 treated SR ATPase. Thus, I suggest that the complicated reaction profile of the initial phase in the FSR ATPase is attributable to the heterogeneity in interactions between the ATPase molecules and PL.

The acceleration of the FSR ATPase activity by ATP itself was first reported by Yamamoto and Tonomura (14) and later confirmed by many investigators (12). Kanazawa et al. (8) indicated that this phenomenon is due to the acceleration of EP formation. But when the solubilized ATPase was used, no acceleration of the ATPase reaction by ATP was observed (Fig. 9) and the value $v/[EP]$ of the solubilized enzyme was much larger than that of FSR, as already reported by Yamada et al. (15) using FSR in the presence of Triton X-100.

Kanazawa et al. (8) found that when the ATPase reaction was stopped by adding sufficient amounts of EGTA and ADP, EP disappeared completely and ATP was formed in an amount equal to the amount of EP which had disappeared at an alkaline pH. Thus, they concluded that EP does not contain bound ADP. On the other hand, Yates et al. (23) recently measured the binding of ADP and ATP to the purified ATPase by a flow-dialysis method, and suggested the existence of EP with bound ADP from the result that the affinity for ADP was similar to that for ATP. Figure 10 clearly indicates that the amount of ADP bound to ATPase during the ATPase reaction

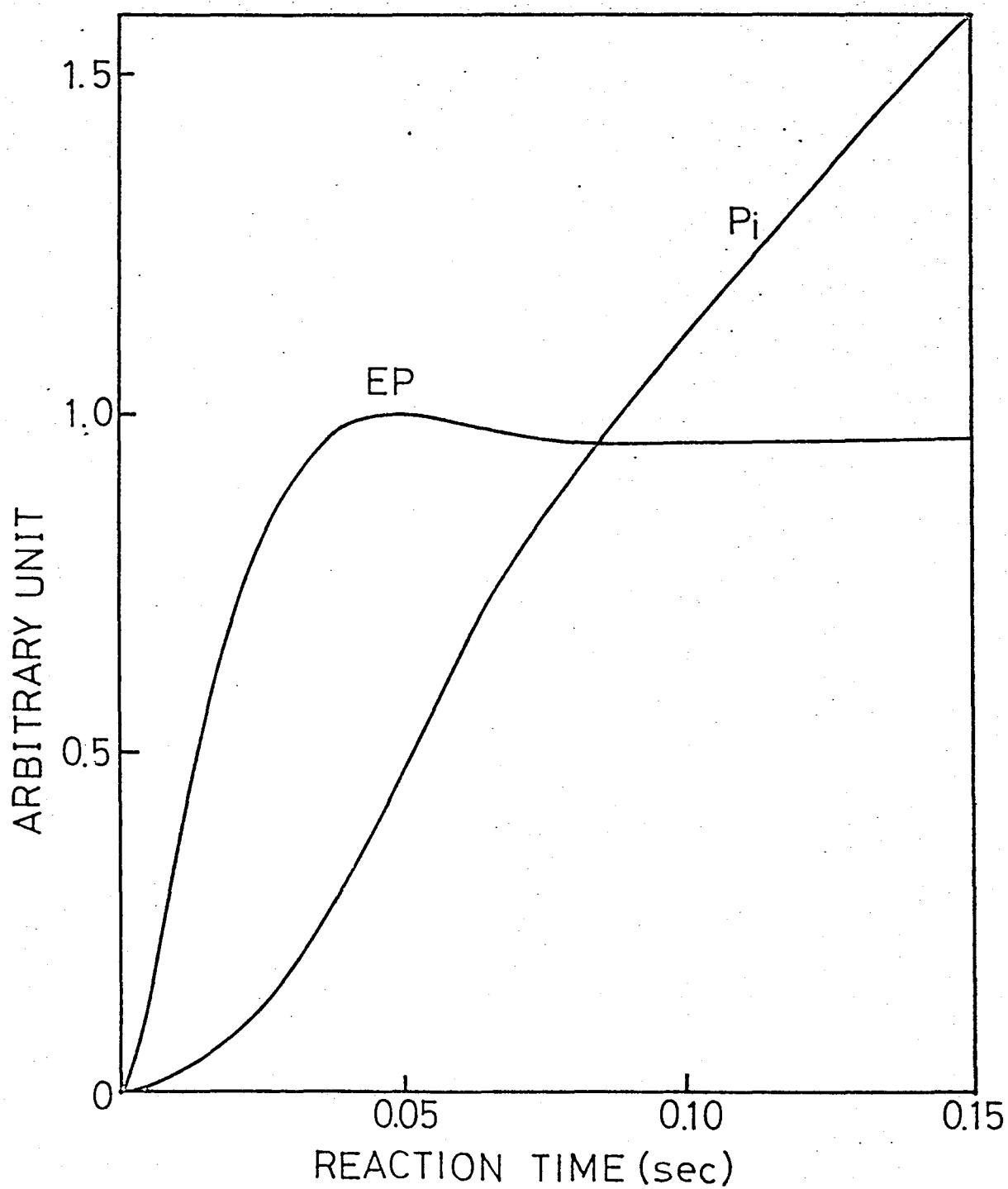


Fig. 11. Analog computer simulation of the time courses of EP formation and P_i liberation during the initial phase.

was negligibly small at least in the steady state and in the presence of CK and CP, thus supporting Kanazawas' conclusion.

* * * * *

This investigation was published in 1978, and it appears in "J.Biochem. 83, 1275-1284."

Fig. 11. Analog computer simulation of the time courses of EP formation and P_i liberation during the initial phase. I assumed that two distinct populations of ATPase (E and E*) exist in a molar ratio of E to *E of 3:1 before addition of ATP, and that the ATPase reactions of *E and E occur as follows: $*E + ATP \xrightleftharpoons{1} *EATP \xrightarrow{2} *EP + ADP \xrightarrow{3} {}^oE + P_i + ADP \xrightarrow{4} E + P_i + ADP$; $E + ATP \xrightleftharpoons{1'} EATP \xrightarrow{2'} EP + ADP \xrightarrow{3'} E + P_i + ADP$. The simulation represents phosphorylation and P_i liberation at 10 μ M ATP. Simulation was done with a Hitachi analog computer ALS-200X. The following set of rate constants was used. $k_1 = k_{1'} = 10^7 \text{ M}^{-1}\text{s}^{-1}$, $k_{-1} = k_{-1'} = 200 \text{ s}^{-1}$, $k_2 = k_{2'} = 150 \text{ s}^{-1}$, $k_3 = 400 [\Delta P_i]/\epsilon \text{ s}^{-1}$, $k_{3'} = 10 \text{ s}^{-1}$, and $k_4 = 2 \text{ s}^{-1}$.

REFERENCES

1. Yamada, S., Yamamoto, T., & Tonomura, Y. (1970) *J. Biochem.* 67, 789-794
2. le Maire, M., Møller, J.V., & Tanford, C. (1976) *Biochemistry* 15, 2336-2342
3. Noda, L., Kubo, S., & Lardy, H. (1955) in Methods in Enzymology (Colowick, S.P. & Kaplan, N.O., eds.) Vol. 2, pp. 605-610, Academic Press, New York
4. Glynn, I.M. & Chappell, J.B. (1964) *Biochem. J.* 90, 965-967
5. Cohn, W.E. & Carter, C.E. (1951) *J. Am. Chem. Soc.* 72, 4273-4275
6. Barman, T.E. & Gutfreund, H. (1964) in Rapid Mixing and Sampling Techniques in Biochemistry (Chance, B., Eisenhardt, R.H., Gibson, Q.H., & Lonberg-Holm, K.K., eds.) pp. 339-344, Academic Press, New York
7. Martin, J.B. & Doty, D.M. (1949) *Anal. Chem.* 21, 965-967
8. Kanazawa, T., Yamada, S., Yamamoto, T., & Tonomura, Y. (1971) *J. Biochem.* 70, 95-123
9. Sumida, M., Kanazawa, T., & Tonomura, Y. (1976) *J. Biochem.* 79, 259-264
10. Inoue, A. & Tonomura, Y. (1974) *J. Biochem.* 76, 755-764
11. Tada, M., Yamamoto, T., & Tonomura, Y. (1978) *Physiol Rev.* 58, 1-79
12. MacLennan, D.H. & Holland, P.C. (1976) in The Enzymes of Biological Membranes (Martonosi, A. ed.) Vol. 3, pp. 221-259, Plenum Press, New York
13. Yamada, S., Sumida, M., & Tonomura, Y. (1972) *J. Biochem.* 72, 1537-1548

14. Yamamoto, T. & Tonomura, Y. (1967) J. Biochem. 62, 558-575
15. Yamada, S., Yamamoto, T., Kanazawa, T., & Tonomura, Y. (1971) J. Biochem. 70, 279-291
16. Froehlich, J.P. & Taylor, E.W. (1975) J. Biol. Chem. 250, 2013-2021
17. Boyer, P.D., de Meis, L., Carvalho, M.G.C., & Hackney, D.D. (1977) Biochemistry 16, 136-140
18. Chiu, V.C.K. & Haynes, D.H. (1977) Biophys. J. 18, 3-22
19. Ikemoto, N. (1976) J. Biol. Chem. 251, 7275-7277
20. Lowe, A.G. & Smart, J.W. (1977) Biochim. Biophys. Acta 481, 695-705
21. Nakamura, Y. & Tonomura, Y. (1978) J. Biochem. 83, 571-583
22. Yamamoto, T. & Tonomura, Y. (1977) J. Biochem. 82, 653-660
23. Yates, D.W. & Duance, V.C. (1976) Biochem. J. 159, 719-728

SUMMARY

The reaction of Ca^{2+} , Mg^{2+} -dependent ATPase [EC 3.6.1.3] of fragmented sarcoplasmic reticulum (FSR) was studied in the pre-steady state, and the following results were obtained.

1. The P_i burst reported by Kanazawa *et al.* [J. Biochem. 70, 95-123 (1971)] was caused by the transition of $v/[\text{EP}]$ after the EP overshoot and the P_i burst observed by Froehlich and Taylor [J. Biol. Chem. 250, 2013-2021 (1975)] had occurred.
2. When the reaction was started by adding Ca^{2+} and AT^{32}P to FSR or AT^{32}P to FSR preloaded with Ca^{2+} , neither the E^{32}P overshoot nor the $^{32}\text{P}_i$ burst were observed.
3. The time course of P_i liberation showed a lag phase and a burst phase, and the apparent rate constant of EP decomposition ($v/[\text{EP}]$) showed a very complicated pattern during the initial phase of the reaction. It increased from the initial value, reached a maximum, then decreased to the steady-state level. This phenomenon was observed more clearly at 4°C than at 20°C .

I also studied the kinetics of solubilized SR ATPase, and obtained the following results.

1. The SR ATPase in the solubilized state showed neither the EP overshoot nor the P_i burst.
2. The double reciprocal plots of the ATPase activity and the amount of EP in the steady state against the ATP concentration gave straight lines over a wide ATP concentration range.
3. The amount of ADP bound to the enzyme during the ATPase reaction was estimated by measuring the amount of ADP remaining in the SR ATPase-ATP system coupled with creatine kinase (CK)

[EC 2.7.3.2] and creatine phosphate (CP). The amount of bound ADP was found to be negligibly small.

I thus concluded that the EP overshoot and the P_i burst which coincided with the transient decay of EP during the initial phase are not caused by the formation of an acid-labile intermediate (E·P) as Froehlich and Taylor suggested, but depend on the enzyme state in the membrane, probably on the interactions of the ATPase with phospholipids (PL). Furthermore, the acceleration of the ATPase reaction by ATP also depends on the enzyme state in the membrane, and the EP intermediate does not contain bound ADP at least in the steady state of the ATPase reaction.

PART II

ADP-Sensitive and -Insensitive Phosphorylated
Intermediates of Solubilized Ca^{2+} , Mg^{2+} -Dependent
ATPase of the Sarcoplasmic Reticulum from
Skeletal Muscle

EXPERIMENTAL PROCEDURE

FSR was prepared from rabbit skeletal muscle, as described previously (1). SSR ATPase was prepared as follows: FSR (120 mg protein) in 20 ml of sucrose buffer containing 0.25 M sucrose, 0.1 M KCl, 0.1 mM CaCl_2 , and 20 mM Tris-TES at pH 7.0 was solubilized with 240 mg of octaethyleneglycol mono-n-dodecylether (C_{12}E_8) (2). The solution was diluted to 120 ml by addition of sucrose-Tween buffer containing 0.25 M sucrose, 50 mM KCl, 0.1 mM CaCl_2 , 0.1 mg/ml Tween 80, and 20 mM Tris-HCl (pH 8.0) at 10°C, then centrifuged at 100,000 x g for 40 min to remove insoluble materials. The clear supernatant was applied to a column (1 x 30 cm) of Whatman DE 52, equilibrated with the sucrose-Tween buffer to remove C_{12}E_8 . ATPase was eluted with 600 ml of a linear gradient of 50 to 550 mM KCl in the sucrose-Tween buffer (Fig. 1). ATPase activity of each fraction was measured in the presence of 5 mM ATP, 90 mM KCl, 5 mM MgCl_2 , 50 μM CaCl_2 , and 50 mM imidazole-HCl at pH 7.0 and 20°C. ATPase [specific activity = about 10 $\mu\text{mol P}_i/(\text{g protein} \cdot \text{s})$; maximum amount of EP = 2 mol/ 10^6 g protein] was eluted at about 0.2 M KCl, and 55,000 dalton protein which did not show ATPase activity was eluted at about 0.4 M KCl. The ATPase fractions (no. 28-41) were essentially free of other proteins according to gel electrophoresis (3), and were stable at least for a week at 0°C. The lipid content of the preparation, determined as inorganic phosphate (4), was about 30 mol per 10^5 g of protein. [γ - ^{32}P]ATP was synthesized enzymatically by the method of Glynn and Chappell (5).

The amount of EP and the ATPase activity in the steady state

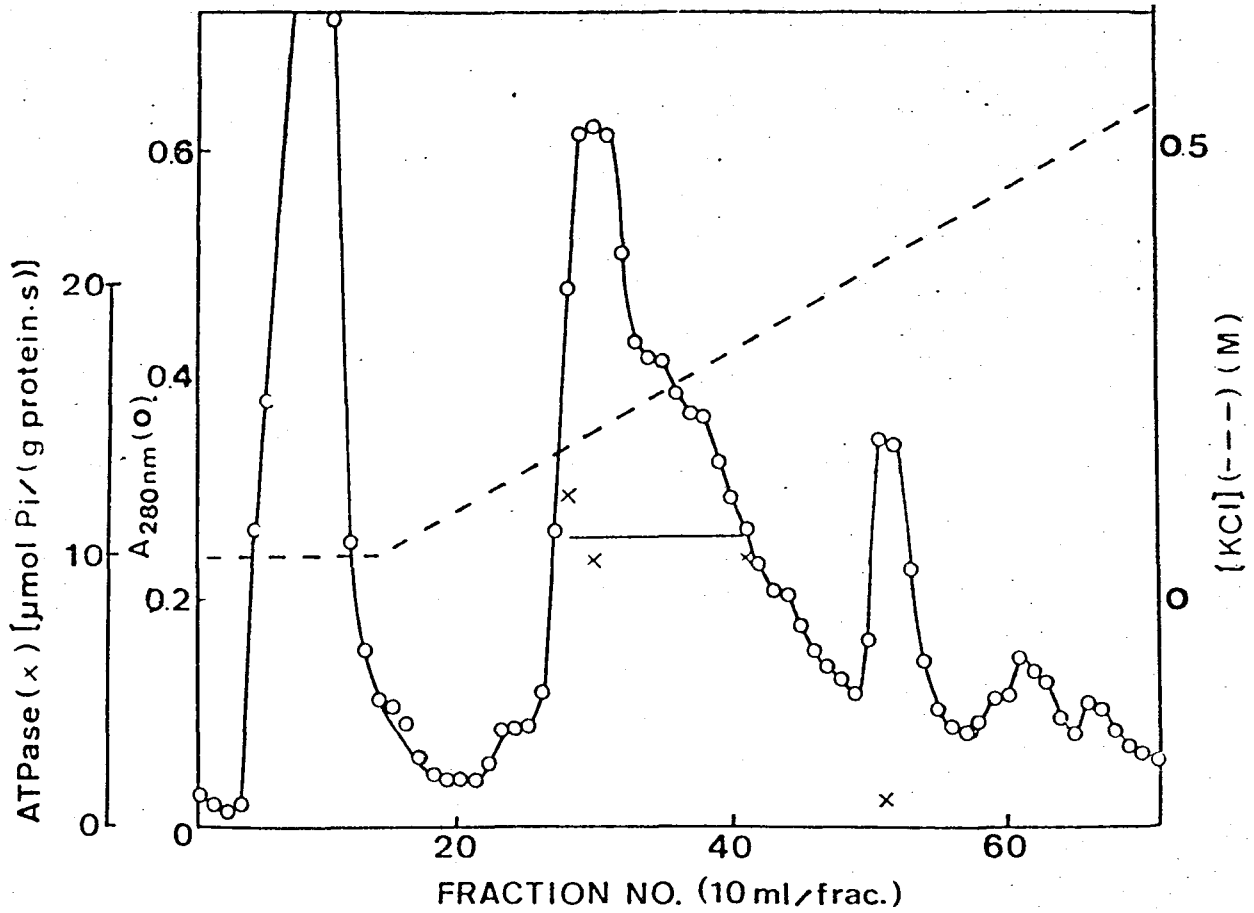
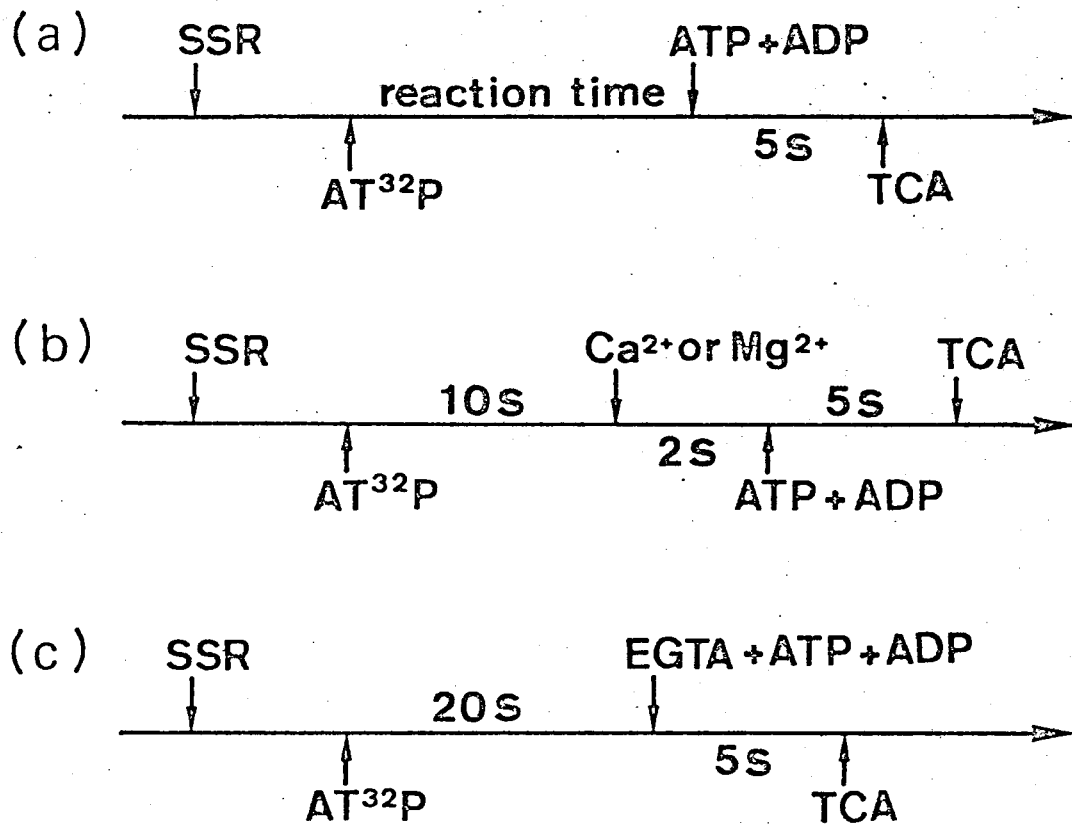


Fig. 1. DEAE-cellulose column chromatography of solubilized sarcoplasmic reticulum. The proteins were eluted with the linear gradient of KCl, indicated by the conductivity (-----). Fractions containing ATPase i.e., those indicated by the horizontal bar, were pooled. For details see text.

were measured, as described previously (6). A Durrum D-133 multi-mixing apparatus (6) and a simple mixing apparatus (7) were used to follow rapid reactions. Various procedures for estimating the amounts of ADP-sensitive and -insensitive EP are outlined in Fig. 2. In the simple methods a, b and c, SSR ATPase was phosphorylated by adding $1\ \mu\text{M}$ AT^{32}P to the reaction mixture containing SSR, KCl, sucrose, MgCl_2 , CaCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C . After appropriate intervals, a solution containing unlabeled ATP and ADP (final concentrations, 1 mM) was added to the reaction mixture (simple method a). The reaction was terminated 5 s later by the addition of 4% TCA. The amounts of E^{32}P remaining and $^{32}\text{P}_i$ liberated after the addition of ATP + ADP were considered to represent the amount of ADP-insensitive EP. The amount of ADP-sensitive EP was obtained by subtracting the amount of ADP-insensitive EP from the total amount of EP just before the addition of ATP + ADP or by measuring the amount of AT^{32}P formed after the addition of ATP + ADP (8). Both methods gave similar values. In the case of the simple method b, a solution containing CaCl_2 or MgCl_2 was added 10 s after the reaction had been started by adding AT^{32}P , then 2 s later ATP + ADP was added to estimate the amounts of the two kinds of EP. In the simple method c, 20 s after the reaction had been started by adding AT^{32}P , a solution containing 1 mM EGTA, 1 mM ATP, and 1 mM ADP was added instead of 1 mM ATP and 1 mM ADP. In the kinetic method, 20 s after the addition of AT^{32}P , a solution containing 1 mM ATP and 1 mM ADP was added to the reaction mixture. After appropriate intervals, 4% TCA was added to terminate the reaction, and the amount of

1. Simple method



2. Kinetic method

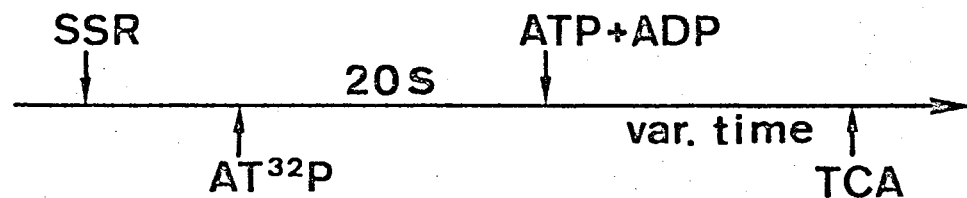


Fig. 2. Various procedures for estimating the amounts of ADP-sensitive and -insensitive EP. For explanation, see text.

AT³²P formed after the addition of 1 mM ATP + 1 mM ADP was measured as described above. From the time course of AT³²P formation, the amount of AT³²P which formed rapidly upon addition of ADP was estimated.

ATPase (1.6 mg protein/ml) was modified with 1 mM MalNEt in 0.25 M sucrose, 0.35 M KCl, 0.1 mM CaCl₂, 0.1 mg/ml Tween 80, and 20 mM Tris-HCl at pH 7.8 and 20°C. After appropriate intervals, the reaction was stopped by adding 10 mM DTT.

Radioactivity was measured with a Beckman LS-150 scintillation counter using 7 ml of liquid scintillation mixture containing 2,5-diphenyloxazole (6 g/liter), 1,4-bis(5-phenyloxazolyl)benzene (0.1 g/liter), and xylene to bring the volume to 1 liter. Protein concentration was determined by the biuret reaction, calibrated by nitrogen determination. The protein concentration of SSR was calculated using the factor, $A_{280} = 1.4 \text{ mg}^{-1}$, that was determined by the biuret method.

RESULTS

Kinetic Properties of SSR ATPase — The time courses of EP formation and P_i liberation in the initial phase of the ATPase reaction were measured. When the reaction was started by adding ATP, the amount of EP increased rapidly with time without a lag phase and reached a steady-state level, while P_i liberation showed a definite pre-steady state which closely corresponded to the period when the amount of EP was increasing (data not shown). After the lag phase, P_i was liberated linearly with time. The dependence on ATP concentration of the rate of ATPase reaction (v_o) and the amount of EP ($[EP]$) in the steady state were measured in the presence of 0.1 M KCl, 0.1 M sucrose, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mg/ml Tween 80, and 50 mM Tris-maleate at pH 7.0 and 10°C. As shown in Fig. 3A, the double reciprocal plots of v_o and $[EP]$ against $[ATP]$ gave straight lines, and the Michaelis constants for both v_o and $[EP]$ were 0.4 μM .

The dependence on ATP concentration of the initial velocity of EP formation (v_f) was also measured under the same conditions as for v_o . Since the time course of EP formation showed a short lag phase (about 20 ms) when the ATP concentration was lower than 2 μM (see Fig. 8), the value of v_f was measured under the conditions where the amount of EP increased linearly with time. As shown in Fig. 3B, the double reciprocal plot of v_f versus $[ATP]$ gave a straight line, and the maximum rate of EP formation and the Michaelis constant (K_f) were 20 $\mu mol/(g \text{ protein} \cdot s)$ and 7.6 μM , respectively.

These results can be explained by a simple mechanism:

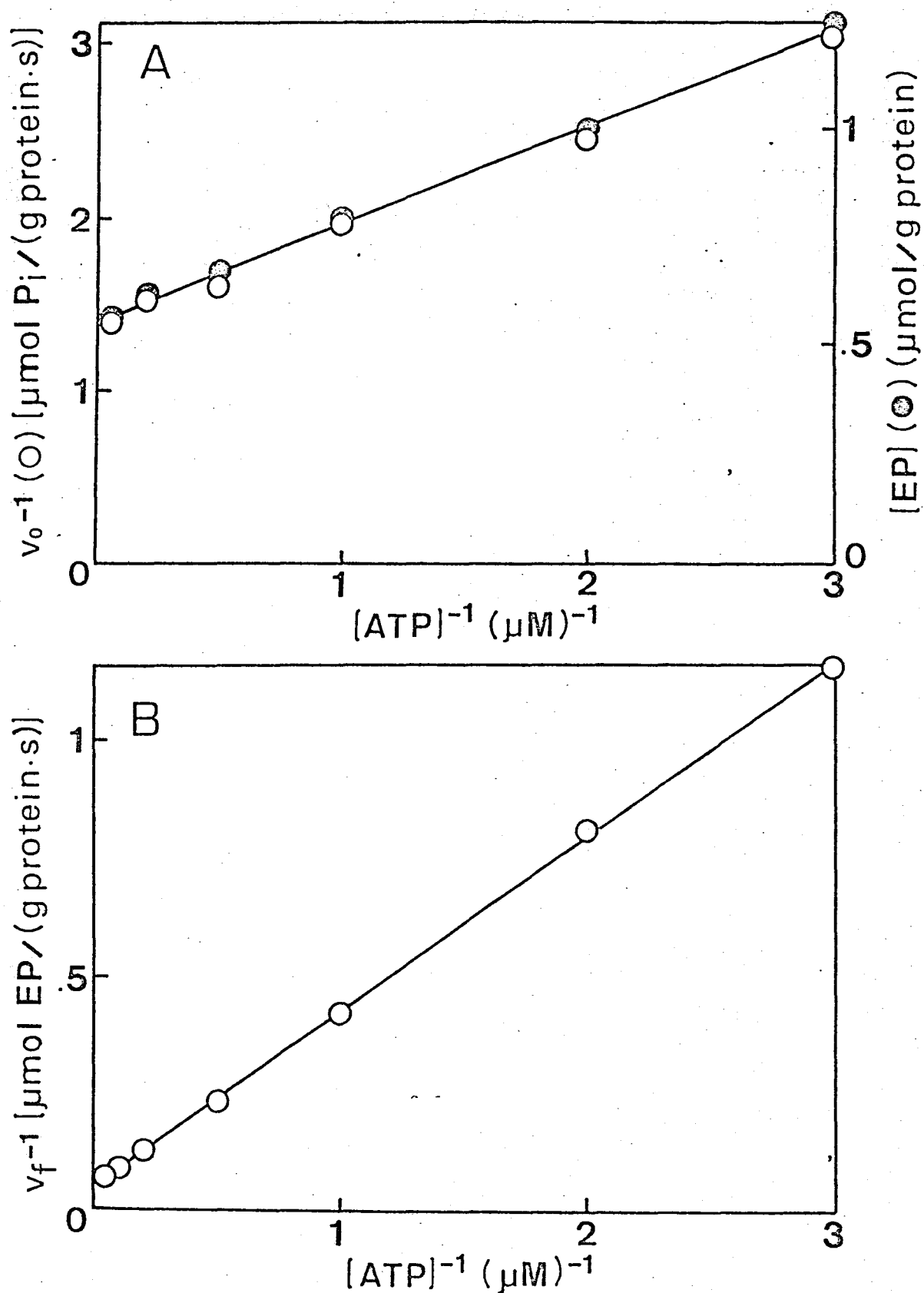


Fig. 3. Dependence on ATP concentration of the ATPase reaction
 A: Double reciprocal plots of the rate of P_i liberation (v_0) and the amount of EP in the steady state against the ATP concentration. The values of v_0 (O) and $[EP]$ (●) were measured in the presence

of 50 $\mu\text{g/ml}$ SSR, 0.1 M KCl, 0.1 M sucrose, 1 mM MgCl_2 , 50 μM CaCl_2 , 0.1 mg/ml Tween 80, and 50 mM Tris-maleate at pH 7.0 and 10°C.

B: Double reciprocal plot of the initial rate of EP formation (v_f) against the ATP concentration. The v_f value was measured under the same conditions described for Fig. 3A, except that the protein concentration was 0.1 mg/ml.

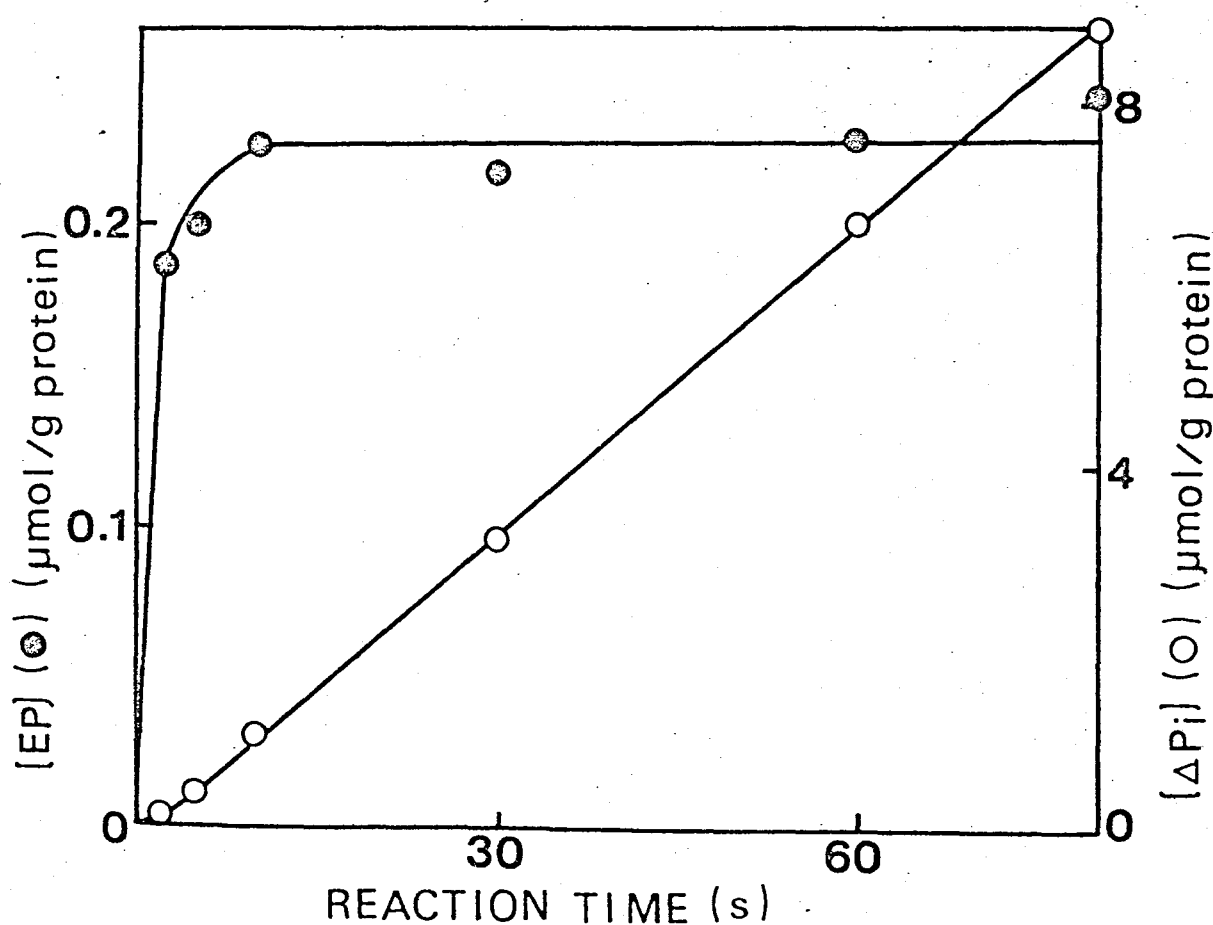
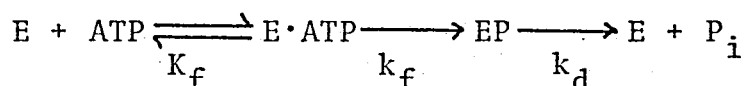


Fig. 4. Initial phase of the SSR ATPase reaction in the presence of ADP. The reaction was started by adding 0.1 mM AT^{32}P to SSR (0.2 mg/ml) in the presence of 1 mM ADP, 0.1 M KCl, 0.1 M sucrose, 2 mM MgCl_2 , 50 μM CaCl_2 , 0.1 mg/ml Tween 80, and 50 mM Tris-maleate at pH 7.0 and 10°C, and the amounts of P_i liberated (○) and EP formed (●) were measured.



since the amount of ADP bound to the enzyme during the ATPase reaction is negligibly small (see Part I). The value of k_d obtained as $v_o/[EP]$ was about 0.4 s^{-1} . The rate of EP formation was much higher than that of its decomposition, and the value of k_f was obtained by dividing the maximum rate of EP formation by the active site concentration which was equal to EP in the presence of sufficient amounts of ATP. The k_f value was about 11 s^{-1} . If it is assumed that the reaction between $E + ATP$ and $E \cdot ATP$ is in rapid equilibrium, K_m is given by ,

$$K_m = K_f \frac{k_d}{k_f + k_d} .$$

The value of K_m thus calculated was about $0.3 \text{ } \mu\text{M}$, which was almost equal to the experimental value, $0.4 \text{ } \mu\text{M}$.

Inhibition of ATPase Activity by ADP — Analysis of the mode of product inhibition is very useful in clarifying the mechanism of an enzyme reaction (9). The effects of ADP on the time courses of EP formation and P_i liberation were measured. The reaction was started by addition of $0.1 \text{ mM AT}^{32}\text{P}$ in the presence of 1 mM ADP , 0.1 M KCl , 0.1 M sucrose , 2 mM MgCl_2 , and $50 \text{ } \mu\text{M CaCl}_2$ at $\text{pH } 7.0$ and 10°C . As shown in Fig. 4, the amount of EP reached the steady-state level at about 10 s after the addition of ATP, and the time course of P_i liberation showed a definite lag phase. I measured the dependence on the ATP concentration of the rate of the ATPase reaction (v_o) and the amount of EP in the steady state. The reaction was started by addition of 100 or $50 \text{ } \mu\text{M ATP}$ in the presence

of 0 to 0.2 mM ADP, 0.1 M KCl, 0.1 M sucrose, 1 mM MgCl_2 , and 50 μM CaCl_2 at pH 7.0 and 10°C. As shown in Fig. 5A, the reciprocal plots of v_0 and [EP] against [ADP] at different ATP concentrations gave straight lines, which intersected at the same point of the horizontal axis, and the K_i value for the non-competitive inhibition was 0.21 mM. However, ADP did not inhibit the decomposition of EP, since the reciprocal plots of both v_0 and [EP] against [ADP] gave the same value of K_i , as shown in Fig. 5A. This was confirmed by the results of Fig. 5B, which showed that the value of v_0 /[EP] was independent of ADP concentration. These results indicated the existence of an EP intermediate which was decreased by the reaction with ADP.

ADP-Sensitive and -Insensitive EP — To clarify whether ATP was formed by the reaction of EP with ADP or not, I performed the following experiments in the presence of 100 mM Tris-HCl at pH 9.0 and 10°C, where EP decomposed very slowly. The phosphorylation reaction was started by addition of 1 μM AT^{32}P to 0.5 mg/ml SSR in the presence of 0.35 M KCl, 0.25 M sucrose, 1 mM MgCl_2 , and 0.1 mM CaCl_2 (Fig. 6). The amount of E^{32}P reached about 1.13 $\mu\text{mol/g}$ protein 5 s later, and further formation of E^{32}P was stopped by the addition of 1 mM unlabeled ATP or 1 mM EGTA. When 1 mM ADP was added simultaneously with ATP (\downarrow), the amount of E^{32}P rapidly decreased and reached the low level of 0.16 $\mu\text{mol/g}$ protein in 10 s. The AT^{32}P concentration in the medium changed inversely with the E^{32}P concentration. After addition of AT^{32}P to SSR, the AT^{32}P concentration rapidly decreased to 0.6 from 2.0 $\mu\text{mol/g}$ protein. On subsequent addition of ADP together with unlabeled ATP, the concentration of AT^{32}P began to increase with a corresponding

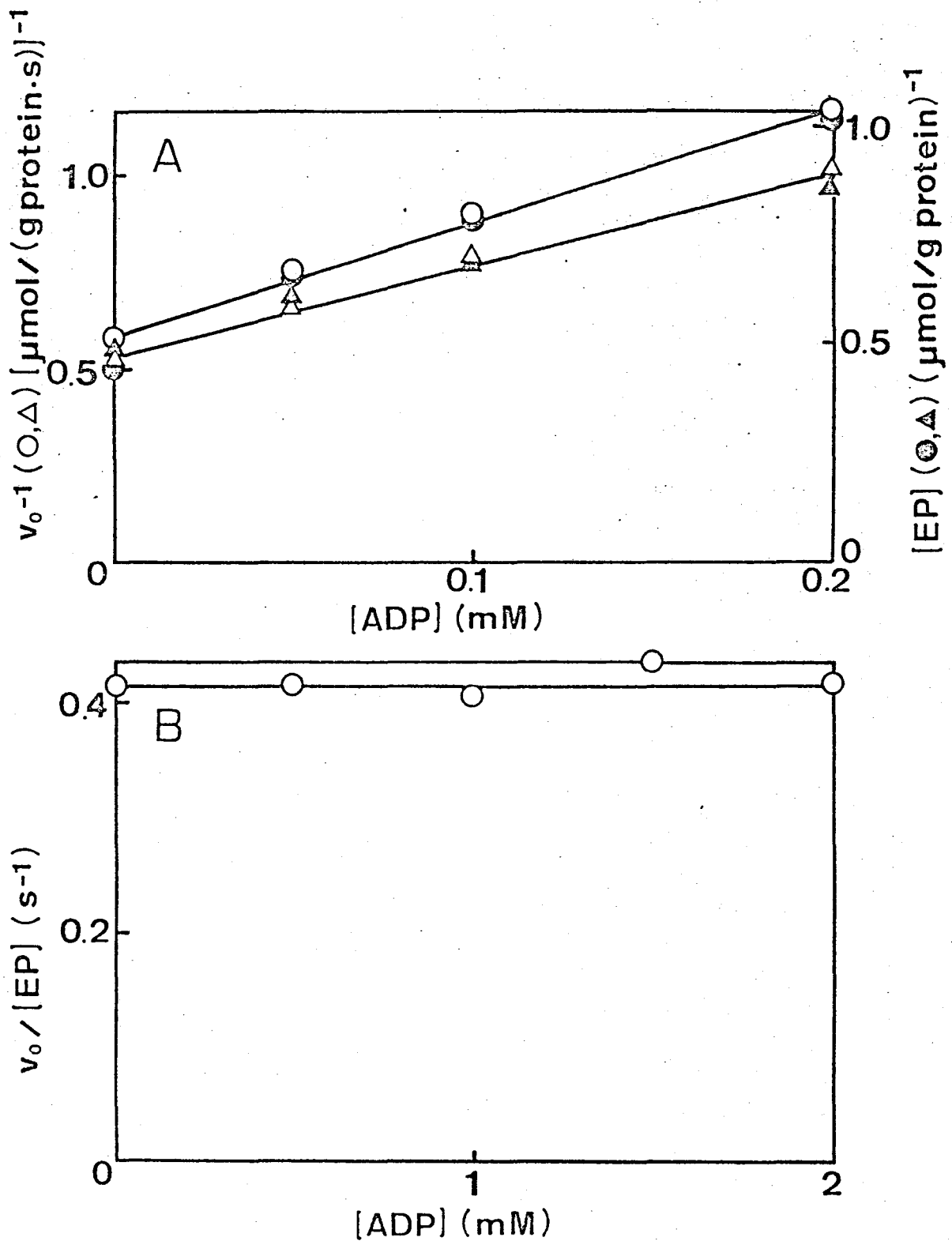


Fig. 5. Dependence on ADP concentration of the rate of ATPase (v_o), the amount of EP in the steady state, and the value of $v_o / [\text{EP}]$.

Fig. 5. Dependence on ADP concentration of the rate of ATPase (v_0), the amount of EP in the steady state, and the value of $v_0/[EP]$. A: Reciprocal plots of the values of v_0 and $[EP]$ against the ADP concentration. The values of v_0 (\circ, Δ) and $[EP]$ (\bullet, \blacktriangle) were measured in the presence of 0.1 mg/ml SSR, 50 (\circ, \bullet) or 100 μM (Δ, \blacktriangle) AT^{32}P , various concentrations of ADP, 0.1 M KCl, 0.1 M sucrose, 1 mM MgCl_2 , 50 μM CaCl_2 , 0.1 mg/ml Tween 80, and 50 mM Tris-maleate at pH 7.0 and 10°C. B: Dependence of $v_0/[EP]$ on ADP concentration. The $v_0/[EP]$ value in the steady state was measured in the presence of 0.2 mg/ml SSR, 100 μM AT^{32}P , various concentrations of ADP, 0.1 M KCl, 0.1 M sucrose, 2.5 mM MgCl_2 , 50 μM CaCl_2 , 0.1 mg/ml Tween 80, and 50 mM Tris-maleate at pH 7.0 and 10°C.

Fig. 6. ATP formation from EP and added ADP both in the presence and absence of Ca^{2+} . SSR (0.5 mg/ml) was phosphorylated with 1 μM AT^{32}P in the presence of 0.35 M KCl, 0.25 M sucrose, 1 mM MgCl_2 , 0.1 mM CaCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 7.0 and 10°C. After 5 s, the formation of E^{32}P was stopped by the addition of 1 mM unlabeled ATP or 1 mM EGTA (\downarrow). With unlabeled ATP, 1 mM ADP (\circ, Δ, \square) or 1 mM ADP + 1 mM EGTA ($\odot, \blacktriangle, \blacksquare$) was added simultaneously, while 1 mM ATP + 1 mM ADP was added 5 s later when the phosphorylation was stopped by EGTA ($\odot, \Delta, \blacksquare$). At intervals after the start of the reaction, the reaction was stopped by adding TCA and the amounts of $^{32}\text{P}_i$ (\circ, \odot, \bullet) E^{32}P ($\Delta, \blacktriangle, \blacktriangle$), and AT^{32}P ($\square, \blacksquare, \blacksquare$) were measured.

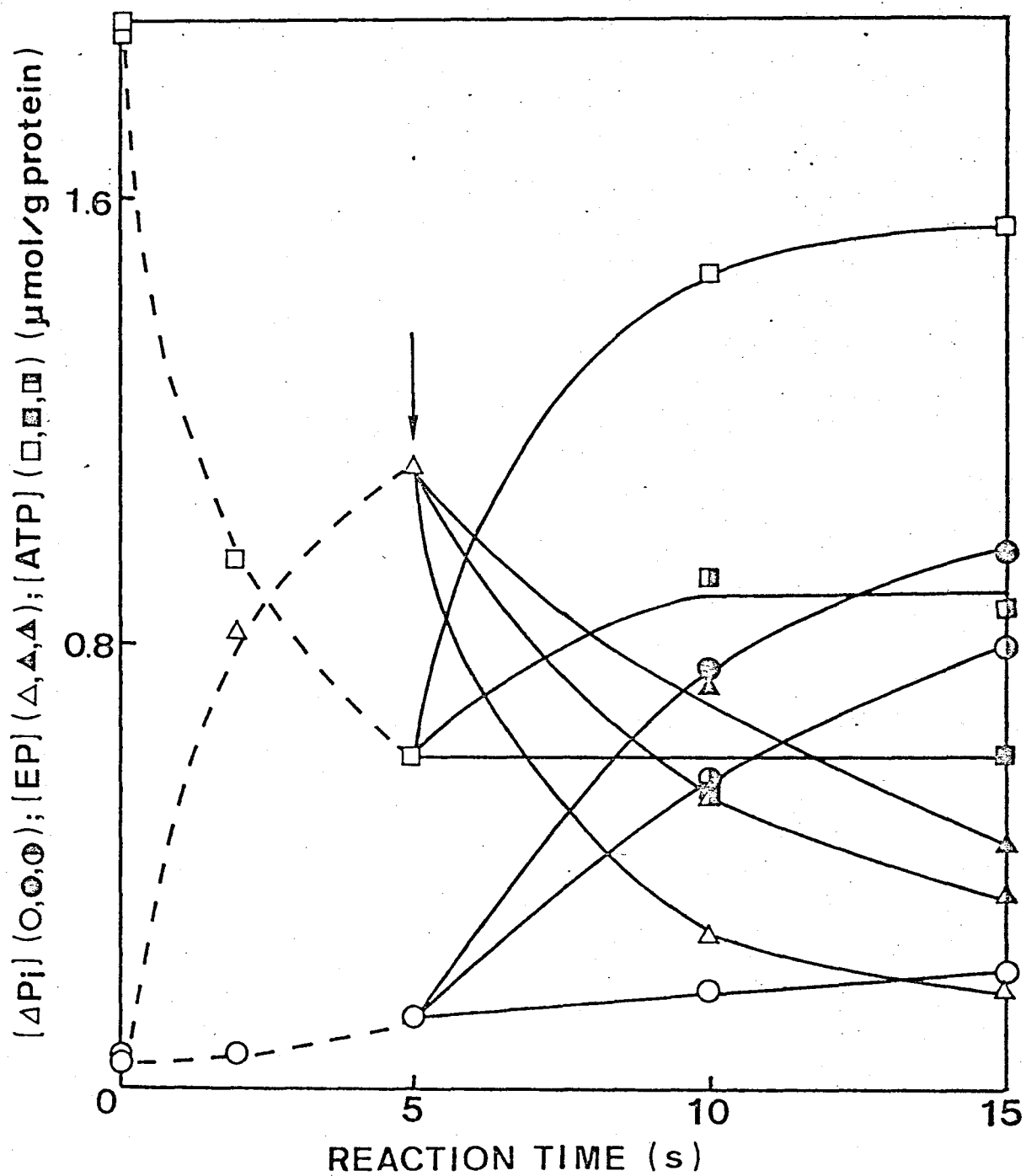


Fig. 6. ATP formation from EP and added ADP both in the presence and absence of Ca^{2+} .

decrease in $E^{32}P$, and reached $1.55 \mu\text{mol/g}$ protein. When 1 mM EGTA was added simultaneously with ATP + ADP, the amount of $E^{32}P$ decreased more slowly than when the reaction was stopped with ATP + ADP and the amount of $AT^{32}P$ formed was only about 30% of that formed upon addition of ATP and ADP. When the phosphorylation reaction was stopped by addition of EGTA and ATP + ADP was added to the reaction mixture 5 s later, no $AT^{32}P$ was formed, and $E^{32}P$ (about $0.7 \mu\text{mol/g}$ protein) was decomposed into $^{32}P_i$ and E. Thus, the reactivity of $E^{32}P$ for ADP was reduced by removing Ca^{2+} with EGTA, and in the absence of Ca^{2+} all the EP was ADP-insensitive.

Figure 7 shows the effect of Ca^{2+} on the ADP sensitivity of EP. The phosphorylation reaction was started by addition of $1 \mu\text{M}$ $AT^{32}P$ to SSR under the same conditions as for Fig. 6. When 2 mM $CaCl_2$ was added simultaneously with 1 mM ATP and 1 mM ADP (\downarrow), almost all the $E^{32}P$ formed reacted with ADP to form $AT^{32}P$. On the other hand, when 1 mM EGTA was added simultaneously with ATP + ADP, only 40% of $E^{32}P$ was converted into $AT^{32}P$.

Figure 8 shows the effects of pH and hydroxylamine on the stability of two kinds of phosphoprotein isolated by TCA treatment (10). SSR was phosphorylated by adding $1 \mu\text{M}$ $AT^{32}P$ in the presence of 0.1 M KCl, 2 mM $CaCl_2$, and 100 mM Tris-HCl at pH 9.0 and 10°C . The reaction was stopped by addition of 4% TCA, and phosphoprotein thus obtained was used as ADP-sensitive EP (cf. Fig. 7). To prepare ADP-insensitive EP, SSR was phosphorylated with $1 \mu\text{M}$ $AT^{32}P$ in the presence of 0.1 M KCl, 0.1 mM $CaCl_2$, and 100 mM Tris-HCl at pH 9.0 and 10°C . After 20 s of reaction, 2 mM EGTA was added, then the reaction was stopped 5 s later by adding 4% TCA (cf. Fig. 6). These two kinds of EP showed the same pH-stability character-

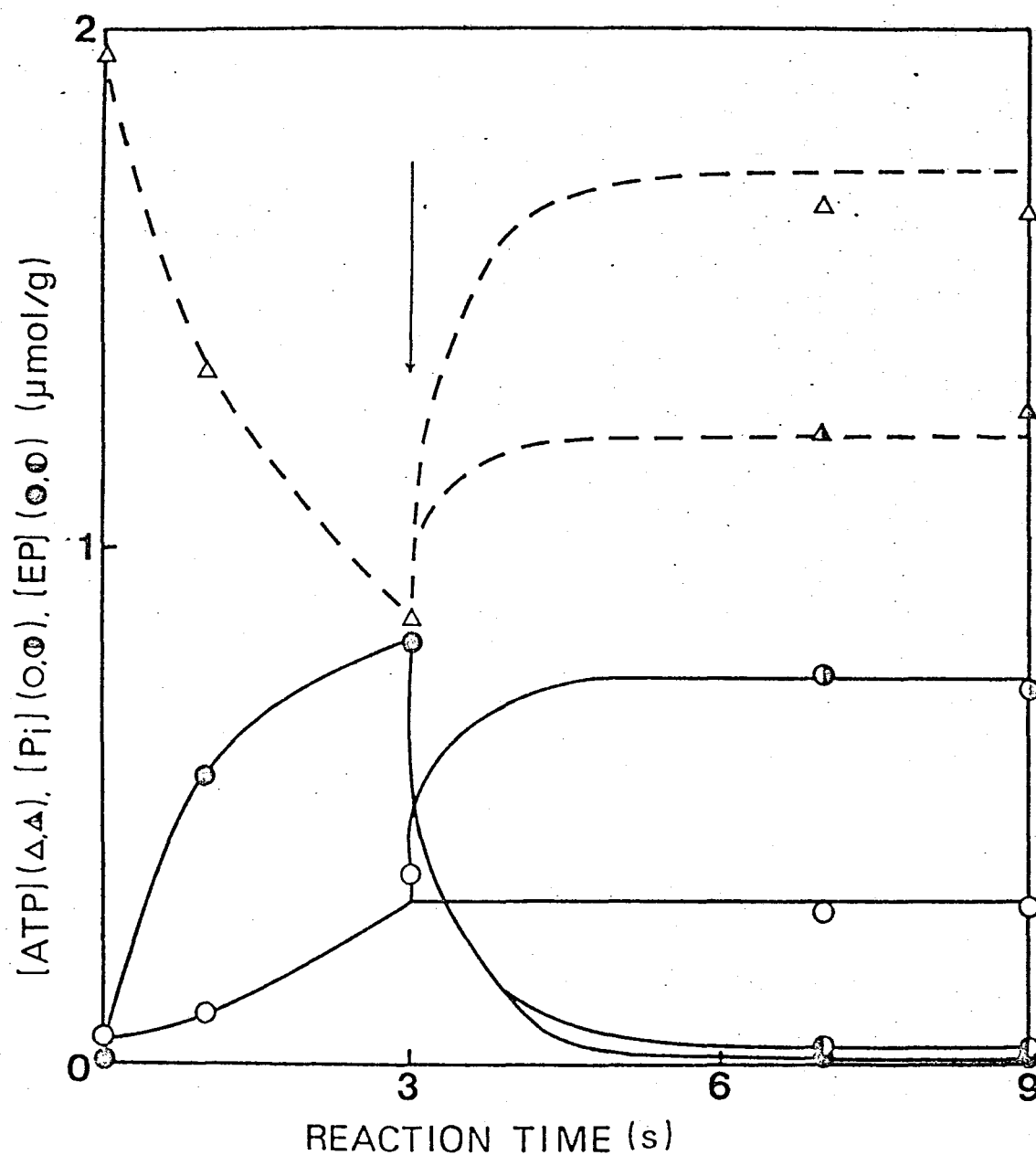


Fig. 7. Effect of Ca^{2+} on the reaction of EP with ADP. SSR (0.5 mg/ml) was phosphorylated with 1 μM AT^{32}P under the same conditions as described for Fig. 6. After 3 s, the formation of E^{32}P was stopped by addition of 1 mM ATP + 1 mM ADP + 2 mM CaCl_2 (○, ○, Δ) or 1 mM ATP + 1 mM ADP + 1 mM EGTA (⊙, ⊙, Δ) (↓). At intervals, the reaction was stopped by addition of TCA, and the amounts of $^{32}\text{P}_i$ (○, ○), E^{32}P (⊙, ⊙) and AT^{32}P (Δ, Δ) were measured.

istics; both were unstable at alkaline pH (Fig. 8A). Furthermore, both rapidly decomposed in the presence of 0.5 M hydroxylamine (Fig. 8B). Thus, it was concluded that the phosphate bonds of the two kinds of EP, which had different reactivities with ADP, were both the acyl phosphate type (10), at least after denaturation with TCA.

Sequential Formation of Two Kinds of EP — Figure 9 shows that the formation of $E^{32}P$ from $AT^{32}P$ was rapidly and completely stopped but the P_i -exchange reaction between $E^{32}P$ and ATP was not induced by the addition of sufficient amounts of unlabeled ATP. The sum of the amounts of $E^{32}P$ and $^{32}P_i$ obtained by stopping the reaction with 1 N HCl (exptl. 1) was equal to the sum of the amounts of $E^{32}P$ and $^{32}P_i$ measured by adding TCA at 15 s after the formation of $E^{32}P$ had been stopped with 1 mM unlabeled ATP at the time indicated as the reaction time (exptl. 2). The $E^{32}P$ formation measured after the HCl stop showed a short lag phase within 20 ms after the start of the reaction, then proceeded linearly with time. After 0.2 s of reaction, the amounts of EP formed and P_i liberated were 0.31 and 0.04 $\mu\text{mol/g}$ protein, respectively.

As shown in Fig. 10, during the steady state about 30% of $E^{32}P$ (exptl. 1) was recovered as the sum of $E^{32}P$ and $^{32}P_i$ when the reaction was stopped by adding 4% TCA at 15 s after the formation of $E^{32}P$ had been stopped by adding 1 mM unlabeled ATP and 1 mM ADP at the time indicated as the reaction time (exptl. 2). Thus, most of the $E^{32}P$ could react with ADP to form $AT^{32}P$. Furthermore, the time course of formation of $E^{32}P$ and $^{32}P_i$ obtained by stopping the reaction with TCA 15 s after adding ATP + ADP showed a definite lag phase. This indicated that during

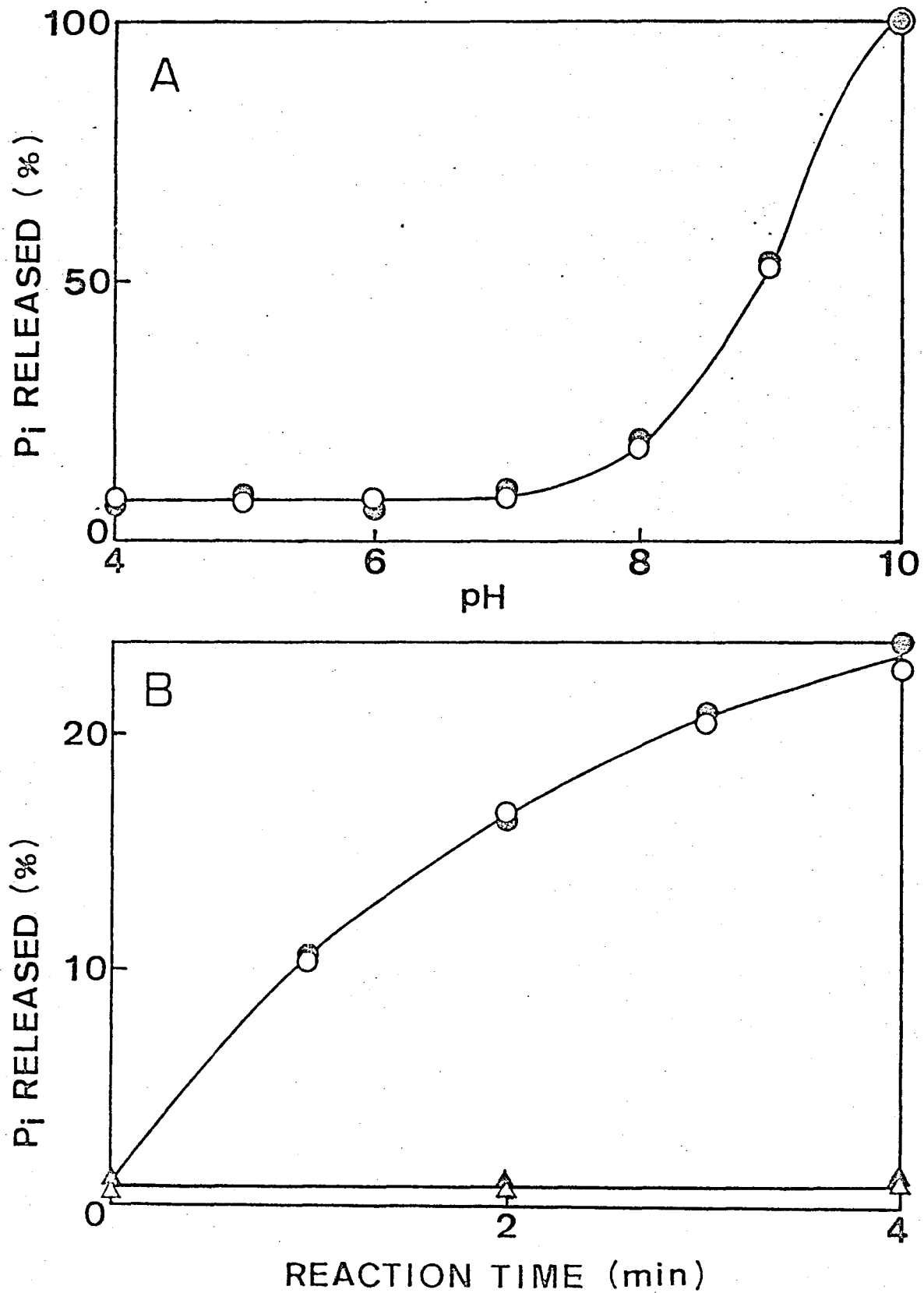


Fig. 8. Chemical characteristics of phosphate bonds of ADP-sensitive and -insensitive EP isolated by TCA treatment. A: pH-dependence of the stability of the two kinds of EP. ADP-sensitive (O) and

-insensitive EP (\odot) were obtained as described in the text, and the amount of P_i released was measured 30 min after incubation of two kinds of EP in 50 mM pH buffer at 20°C. B: Acceleration by hydroxylamine of the decomposition of the two kinds of EP. ADP-sensitive (\circ, Δ) and -insensitive EP (\odot, \blacktriangle) were incubated with 0.5 M hydroxylamine (\circ, \odot) or 0.5 M KCl (Δ, \blacktriangle) in the presence of 50 mM Tris-maleate at pH 5.5 and 20°C.

Fig. 9. Time courses of $E^{32}P$ formation and $^{32}P_i$ liberation during the initial phase when the reaction was stopped with 1 N HCl or 1 mM unlabeled ATP. SSR (0.15 mg/ml) was phosphorylated with 0.5 μ M $AT^{32}P$ in the presence of 0.1 M KCl, 0.1 M sucrose, 1 mM $MgCl_2$, 50 μ M $CaCl_2$, 0.1 mg/ml Tween 80, and 50 mM Tris-maleate at pH 7.0 and 10°C. After appropriate intervals, the formation of $E^{32}P$ was stopped with 1 N HCl (\circ, \odot). It was also stopped with 1 mM unlabeled ATP (Δ, \blacktriangle), then the reaction was stopped 15 s later with 4% TCA. The amounts of $^{32}P_i$ liberated (\circ, Δ) and $E^{32}P$ formed (\odot, \blacktriangle) were measured.

Fig. 10. Time courses of $E^{32}P$ formation and $^{32}P_i$ liberation during the initial phase when the reaction was stopped with 1 N HCl or 1 mM unlabeled ATP + 1 mM ADP. SSR (0.15 mg/ml) was phosphorylated with 0.5 μ M $AT^{32}P$ under the same conditions as described for Fig. 9. After appropriate intervals, the formation of $E^{32}P$ was stopped with 1 N HCl (\circ, \odot). It was also stopped with 1 mM unlabeled ATP + 1 mM ADP (Δ, \blacktriangle), then the reaction was stopped 15 s later with 4% TCA. The amounts of $^{32}P_i$ liberated (\circ, Δ) and $E^{32}P$ formed (\odot, \blacktriangle) were measured.

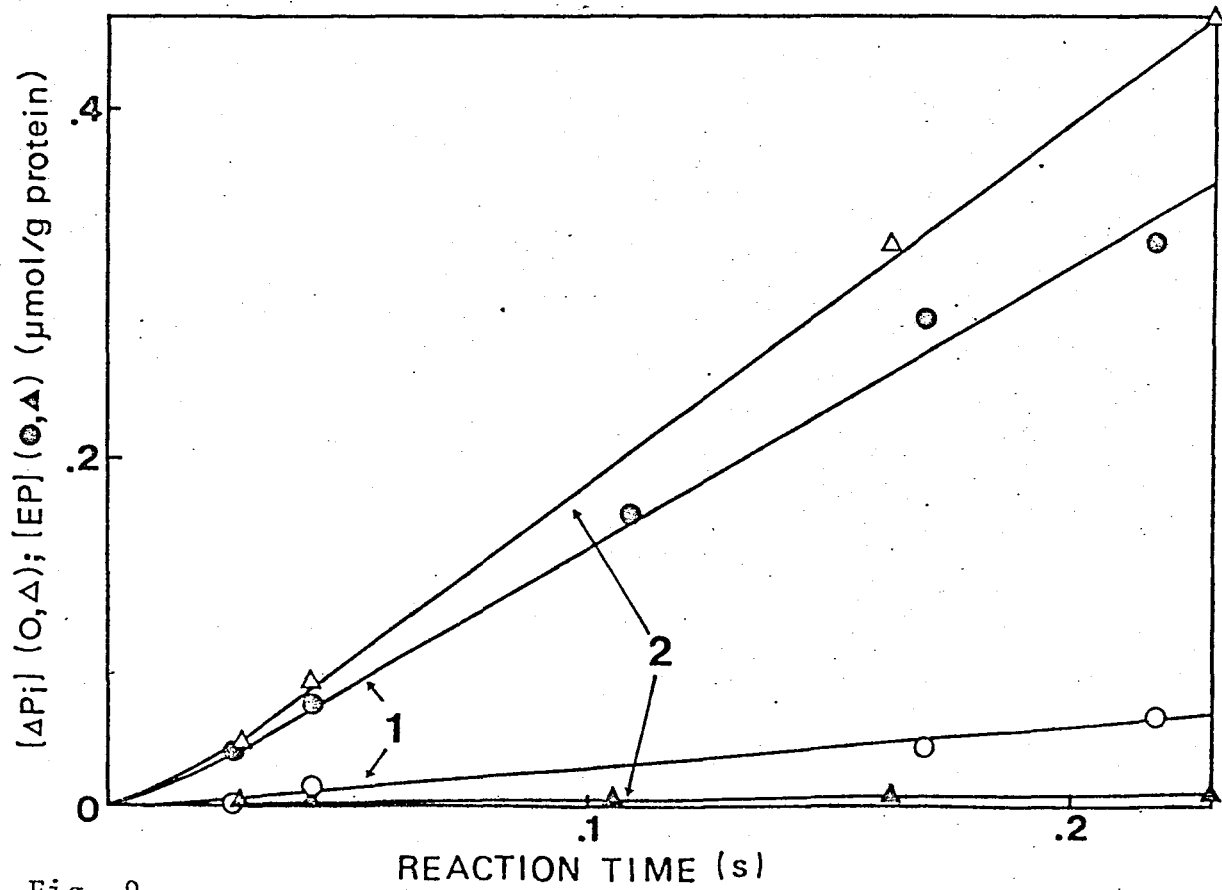


Fig. 9.

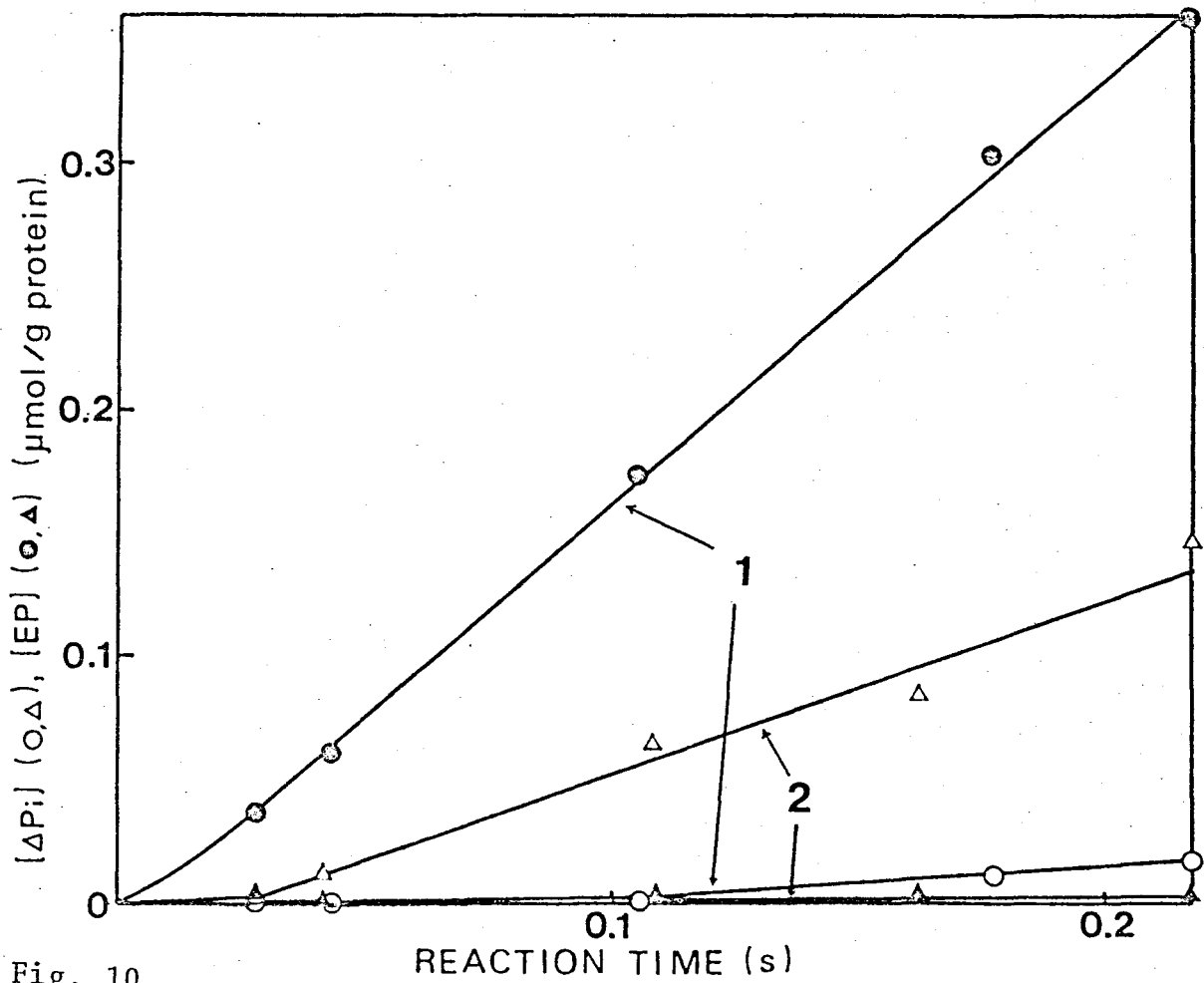
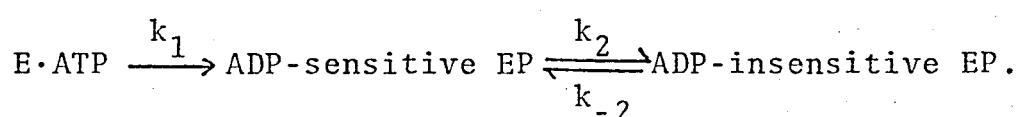


Fig. 10

the initial phase, ADP-sensitive EP was produced, then ADP-insensitive EP was formed.

The time courses of formation of the two kinds of EP were measured in 100 mM Tris-HCl at pH 9.0 and 10°C, where the EP decomposition was very slow (Fig. 11). The reaction was started by adding 1 μ M AT^{32}P to SSR (0.4 mg/ml) in 0.3 M KCl, 0.2 M sucrose, 10 mM MgCl_2 , and 0.1 mM CaCl_2 . After appropriate intervals, the reaction was stopped by addition of 4% TCA, and the total amount of E^{32}P was measured. The amount of ADP-insensitive EP at each time was measured by the simple method as described in "EXPERIMENTAL PROCEDURE." As shown in Fig. 11, the total amount of EP increased with time and reached the steady-state level (about 0.8 μ mol/g protein) within 3 s after the start of the reaction. On the other hand, the time course of formation of ADP-insensitive EP showed a definite lag phase (about 0.5 s), and the amount of ADP-insensitive EP reached the steady-state level about 3 s after the start of the reaction. The amount of ADP-sensitive EP, which was obtained by subtracting the ADP-insensitive EP from the total EP, increased with time, and reached the steady state level (60% of total EP) within 1 s after the start of the reaction. These results indicate that ADP-sensitive EP is formed first then converted into ADP-insensitive EP. Simulation on a Hitachi analog computer ALS-200X of the formation of the two kinds of EP also supported the idea of their sequential formation. I assumed the following reaction steps for their formation:



The k_1 value was found to be 1.1 s^{-1} from the time course of

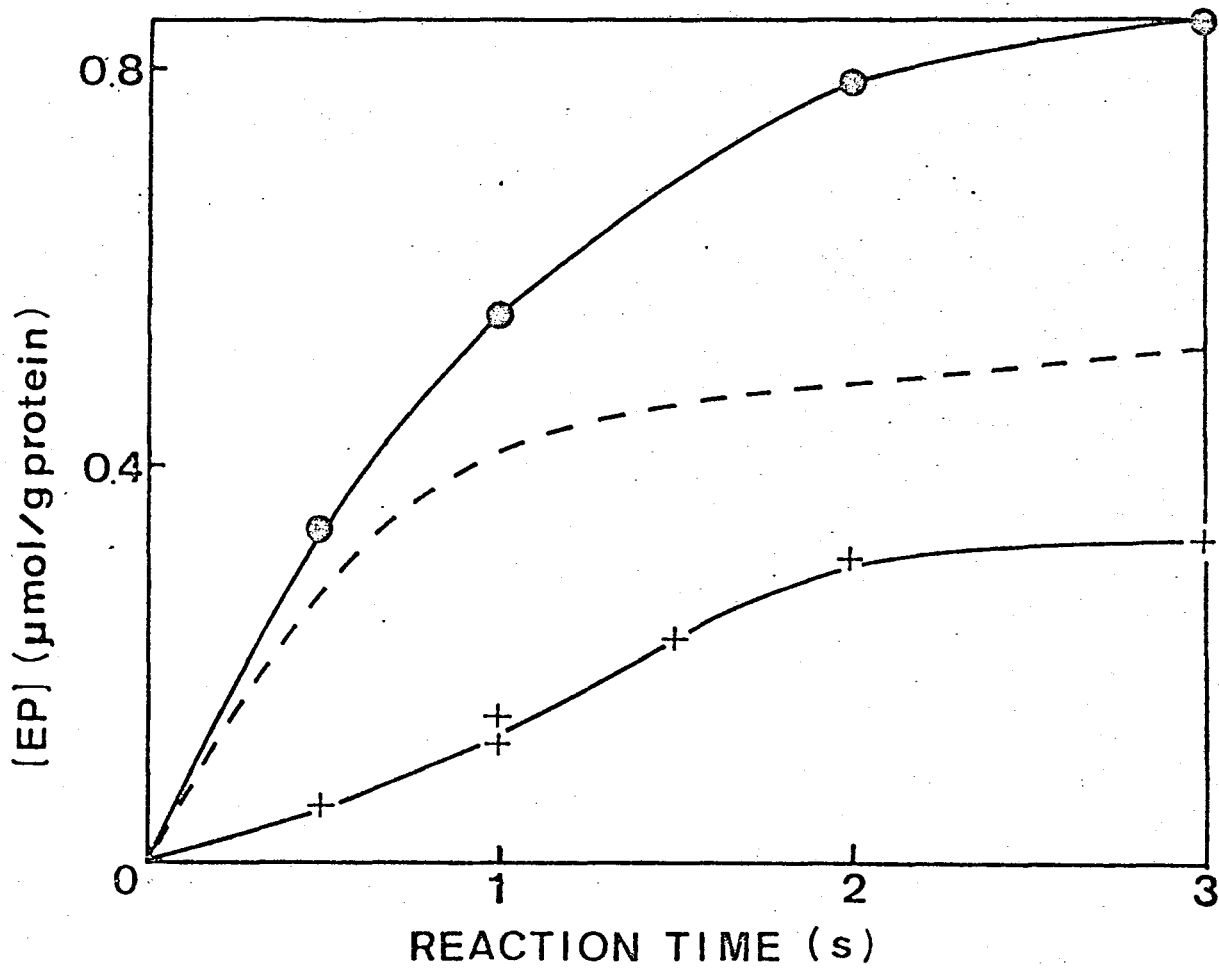


Fig. 11. Time courses of formation of ADP-sensitive and -insensitive EP. SSR (0.4 mg/ml) was phosphorylated with $1 \mu\text{M}$ AT^{32}P in the presence of 0.3 M KCl, 0.2 M sucrose, 10 mM MgCl_2 , 0.1 mM CaCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C . After appropriate intervals, the reaction was stopped by the addition of 4% TCA, and the total amount of EP (●) was measured. The amount of ADP-insensitive EP (+) at each time was measured by the simple method a, and the time course of formation of ADP-sensitive EP (— —) was obtained by subtracting the ADP-insensitive EP from the total EP at each time.

formation of total EP in Fig. 11. The k_2 value was 0.5 s^{-1} (cf. Fig. 18). The best fit to the time courses of formation of the two kinds of EP obtained by computer simulation, was when a 0.6 s^{-1} was used as k_{-2} .

Conversion of Two Kinds of EP by Divalent Cations — Figure 12 shows the dependence of the ratio of ADP-sensitive EP to total EP on CaCl_2 and MgCl_2 concentrations. In the experiments shown in Fig. 12A, SSR (0.4 mg/ml) was phosphorylated with AT^{32}P in the presence of 20 mM MgCl_2 and 0.1 mM CaCl_2 , and 10 s later appropriate amounts of CaCl_2 were added to the reaction mixture, then the amount of ADP-sensitive EP was estimated by the simple method b described in "EXPERIMENTAL PROCEDURE." The ratio increased with an increase in the CaCl_2 concentration, while the total amount of EP at steady state was unaffected by CaCl_2 and was about $1.6 \mu\text{mol EP/g protein}$. In the experiments shown in Fig. 12B, SSR (0.4 mg/ml) was phosphorylated with AT^{32}P in the presence of 1 mM MgCl_2 and 0.1 mM CaCl_2 , and 10 s later appropriate amounts of MgCl_2 were added to the reaction mixture, then the amount of ADP-sensitive EP was estimated by the simple method b. The ratio of ADP-sensitive EP to total EP decreased when the MgCl_2 concentration was increased. The amount of EP at steady state was unaffected by MgCl_2 and was about $1.7 \mu\text{mol/g protein}$. These results indicated that ADP-sensitive EP was a complex of EP with Ca^{2+} and ADP-insensitive EP was a complex of EP with Mg^{2+} .

Figure 13 shows the Hill plots of $\{ [\text{total EP}]/[\text{ADP-insensitive EP}] - 1 \}$ against $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$. Both Hill coefficients for Ca^{2+} and Mg^{2+} were 2. The value of $K_{\text{Mg}}/K_{\text{Ca}}$ obtained from the Hill plot against $[\text{Mg}^{2+}]$ was 52.7, which agreed well with the

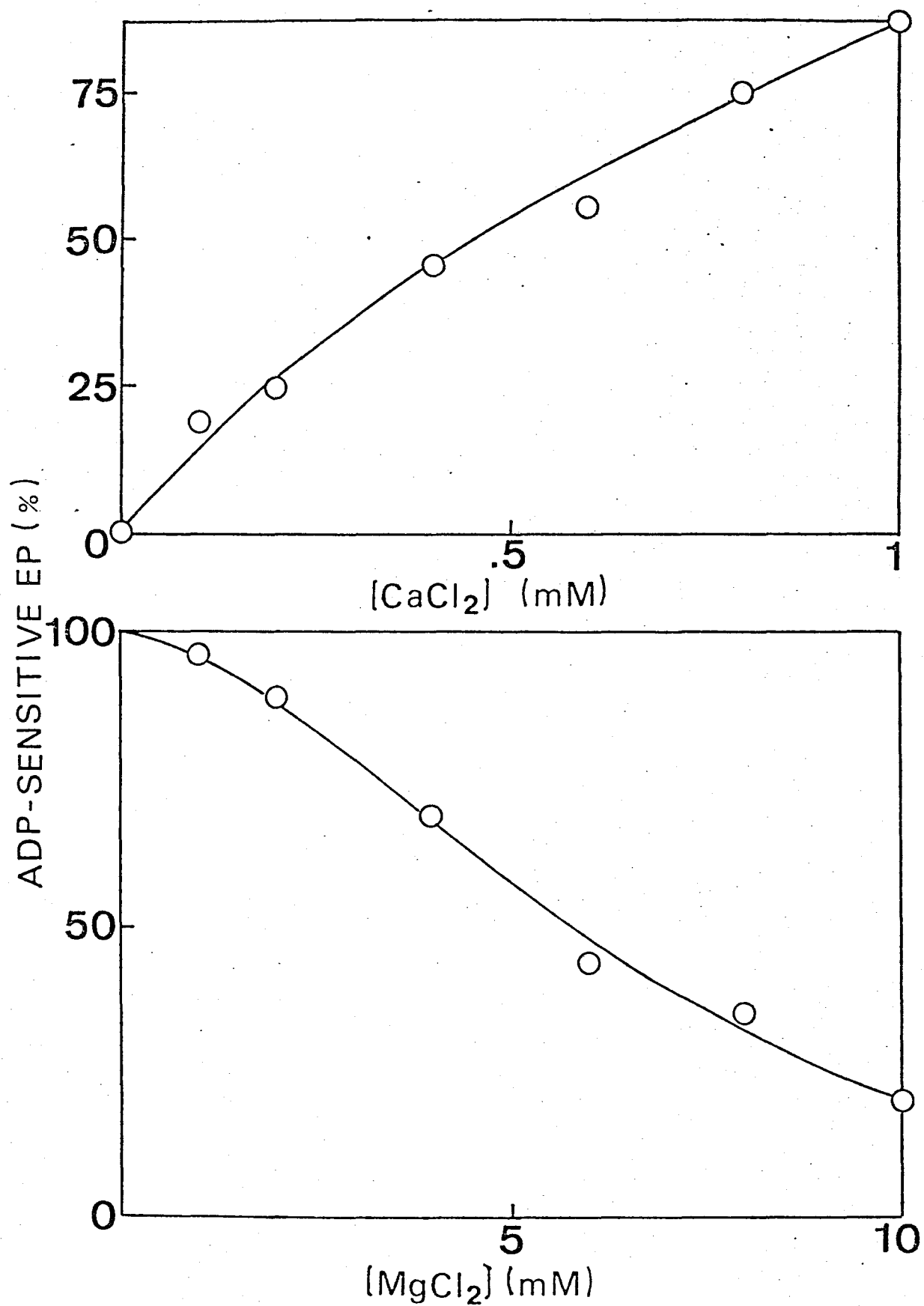


Fig. 12. Dependence of the ratio of ADP-sensitive EP to total EP on CaCl_2 and MgCl_2 concentrations.

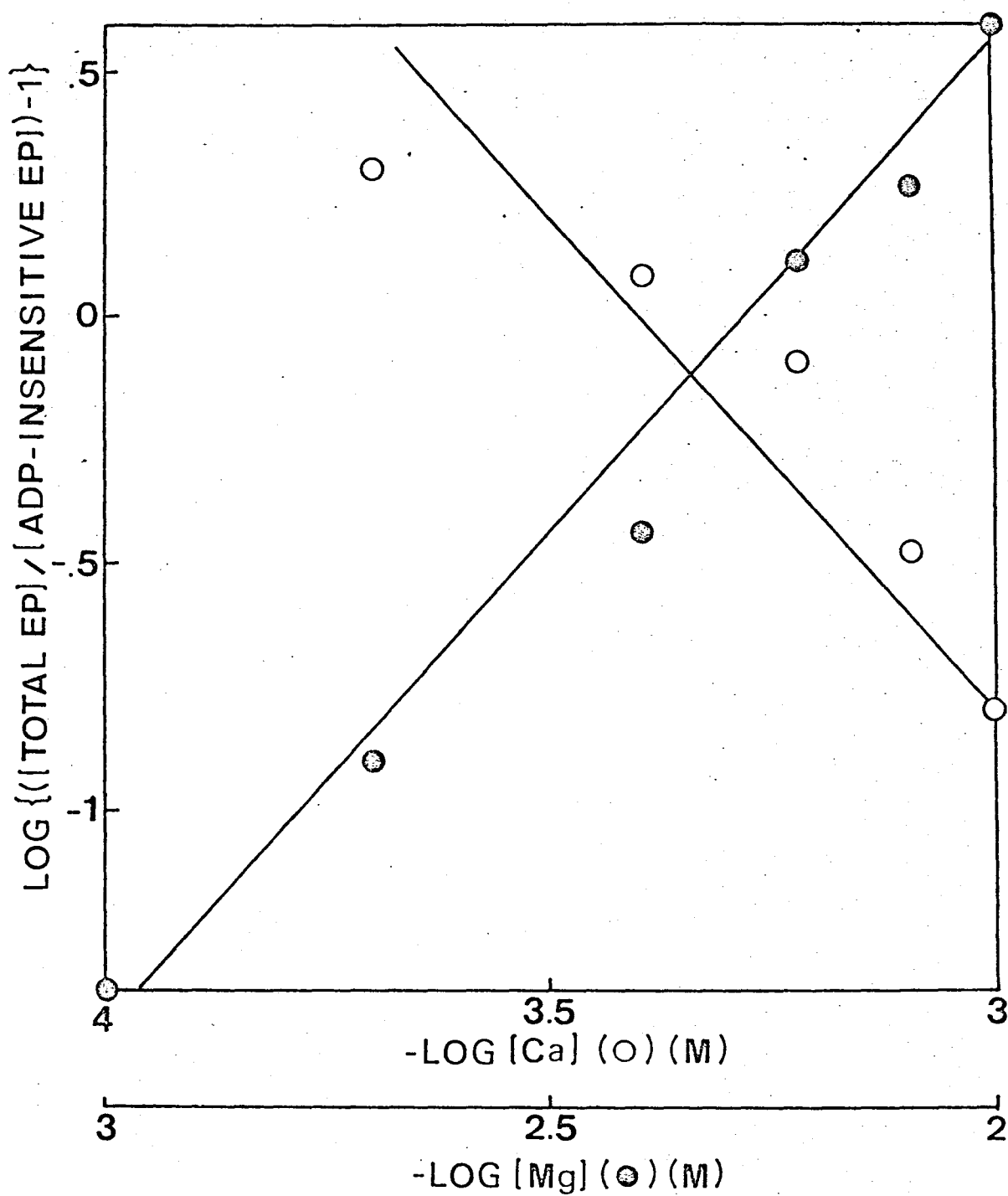


Fig. 13. Hill plots of $\{ ([\text{total EP}] / [\text{ADP-insensitive EP}]) - 1 \}$ against $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$. The value of $[\text{total EP}] / [\text{ADP-insensitive EP}]$ was calculated from the results given in Fig. 12.

Fig. 12. Dependence of the ratio of ADP-sensitive EP to total EP on CaCl_2 and MgCl_2 concentrations. A: Dependence of the ratio on CaCl_2 concentration. SSR (0.4 mg/ml) was phosphorylated with 1 μM AT^{32}P in the presence of 0.3 M KCl, 0.2 M sucrose, 20 mM MgCl_2 , 0.1 mM CaCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C. Ten seconds later, appropriate amounts of CaCl_2 were added, then the amount of ADP-sensitive EP was estimated by the simple method b. B: Dependence of the ratio on MgCl_2 concentration. SSR (0.4 mg/ml) was phosphorylated with 1 μM AT^{32}P under the same conditions described for Fig. 12A, except that the MgCl_2 concentration was 1 mM. Ten seconds later, appropriate amounts of MgCl_2 were added, then the amount of ADP-sensitive EP was estimated.

Fig. 14. Effects of CaCl_2 concentration on the time course of AT^{32}P formation from E^{32}P and ADP and the value of $v_0/[\text{EP}]$. A: Time course of AT^{32}P formation by the reaction of E^{32}P with ADP in the presence of various concentrations of CaCl_2 . The reaction was started by adding 1 μM AT^{32}P to SSR (0.4 mg/ml) in 0.15 M KCl, 0.1 M sucrose, 1 mM MgCl_2 , various concentrations of CaCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C, and 20 s later, the formation of E^{32}P was stopped with 1 mM unlabeled ATP + 1 mM ADP, and the time courses of AT^{32}P formation in the presence of 5 (\times), 30 (∇), 55 (\blacksquare), 85 (\blacktriangle) and 155 (\bullet) μM CaCl_2 were measured. B: Dependence of the value of $v_0/[\text{EP}]$ and the amount of ADP-insensitive EP on CaCl_2 concentration. The reaction was performed under the same conditions described for Fig. 14A. The values of v_0 and $[\text{EP}]$ were measured by adding 4% TCA instead of ATP + ADP 20 s after the start of the reaction, and the amount of ADP-insensitive EP was measured by the kinetic method.

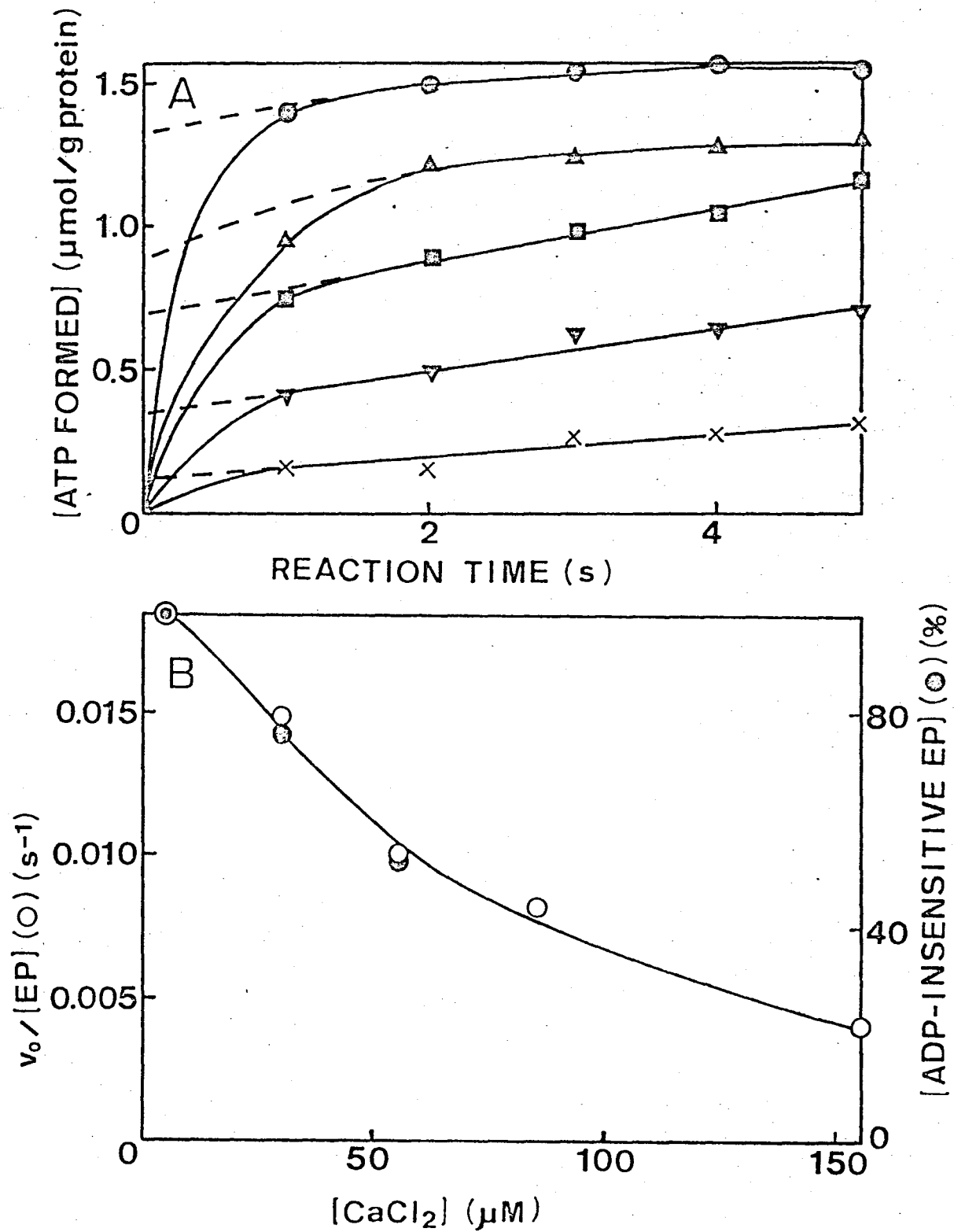


Fig. 14. Effects of CaCl_2 concentration on the time course of AT^{32}P formation from E^{32}P and ADP and the value of $v_0/[\text{EP}]$.

value of 50.2 obtained from the Hill plot against $[Ca^{2+}]$.

In the above experiments, the amount of ADP-insensitive EP was estimated as the amount of $E^{32}P$ remaining and $^{32}P_i$ liberated 5 s after the addition of ATP + ADP. However, as shown in Fig. 14A, the time course of $AT^{32}P$ formation upon adding ATP + ADP to $E^{32}P$ consisted of fast and slow phases. Therefore, the amount of $AT^{32}P$ formed in the fast phase was taken as the amount of ADP-sensitive EP in the following experiments. Thus, in the experiments shown in Fig. 14A, SSR (0.4 mg/ml) was phosphorylated with $1 \mu M AT^{32}P$ in the presence of 1 mM $MgCl_2$ and various concentrations of $CaCl_2$, then 1 mM ATP + 1 mM ADP was added 20 s after the addition of $AT^{32}P$ and the time course of $AT^{32}P$ formation was measured. The amounts of EP and the ATPase activity at steady state were also measured by stopping the reaction with TCA instead of ATP + ADP. As shown in Fig. 14B, the apparent rate constant of EP decomposition ($v_o/[EP]$) decreased with an increase in $CaCl_2$ concentration. The amount of ADP-insensitive EP obtained by the kinetic method was proportional to $v_o/[EP]$ over the whole range of $CaCl_2$ concentrations tested. In this figure, the amount of ADP-insensitive EP is given as a percentage, since the amount of total EP was independent of $CaCl_2$ concentration, as mentioned above.

In the experiments shown in Fig. 15A, SSR (0.4 mg/ml) was phosphorylated with $1 \mu M AT^{32}P$ in the presence of 0.1 mM $CaCl_2$ and various concentrations (1 - 5 mM) of $MgCl_2$, then 1 mM ATP + 1 mM ADP was added to the reaction mixture 20 s later. The ATPase activity at steady state decreased slightly with an increase in the concentration of $MgCl_2$, while the amount of total EP was

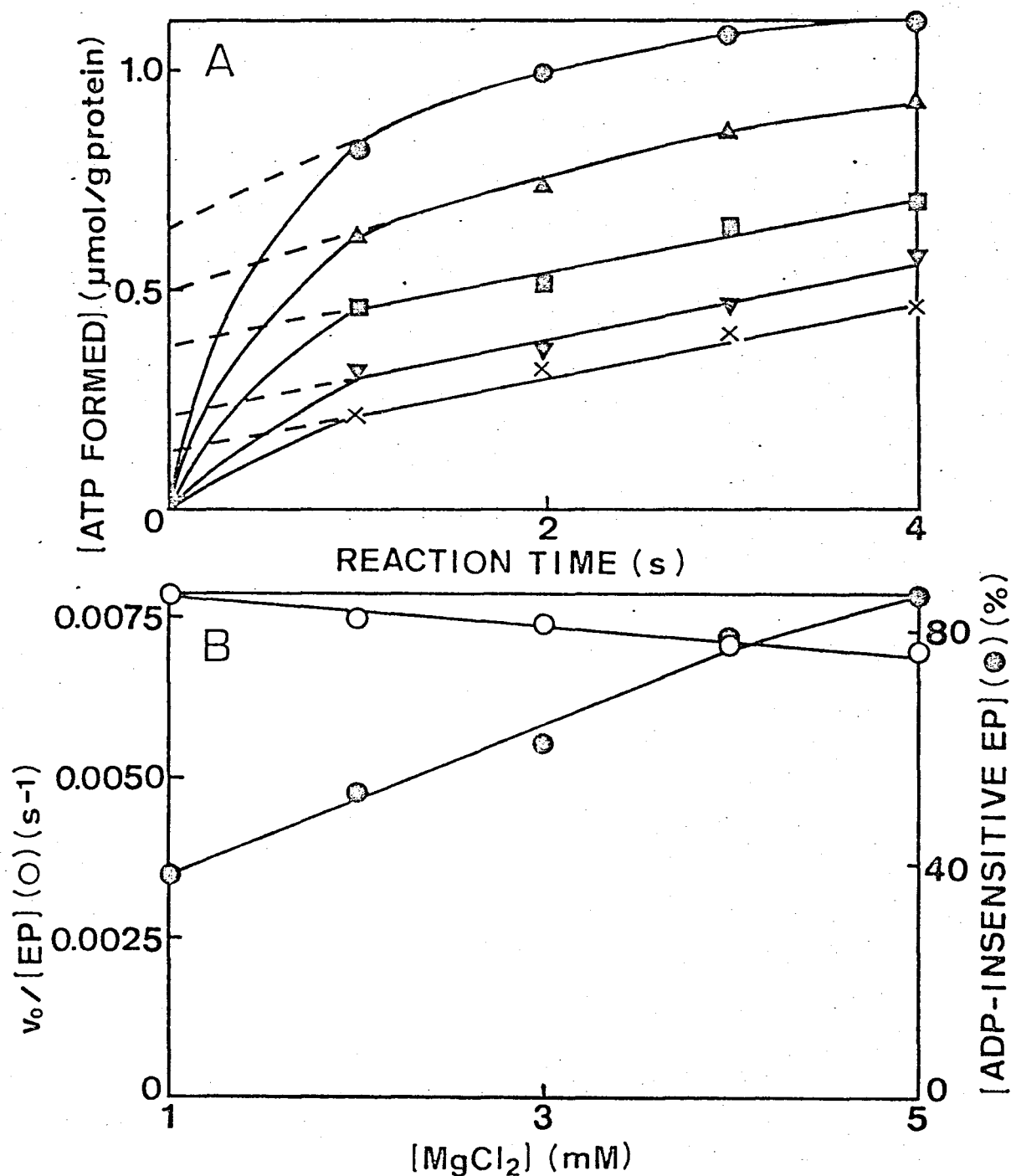


Fig. 15. Effect of MgCl_2 concentration on the time course of AT^{32}P formation from E^{32}P and ADP and the value of $v_0/[\text{EP}]$. A: Time course of AT^{32}P formation by the reaction of E^{32}P with ADP in the presence of various concentrations of MgCl_2 . The reaction was started by adding $1 \mu\text{M}$ AT^{32}P to SSR (0.4 mg/ml) in 0.15 M KCl, 0.1 M sucrose, 0.1 mM CaCl_2 , various concentrations of MgCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C , then the formation of E^{32}P was stopped 20 s later with 1 mM unlabeled ATP +

1 mM ADP, and the time courses of AT^{32}P formation in the presence of 1 (\odot), 2 (Δ), 3 (\blacksquare), 4 (∇), 5 (\times) mM MgCl_2 were measured.

B: Dependence of the value of $v_0/[\text{EP}]$ and the amount of ADP-insensitive EP on MgCl_2 concentration. The reaction was performed under the same conditions described for Fig. 15A. The values of v_0 and $[\text{EP}]$ were measured by adding 4% TCA, instead of ATP + ADP, 20 s after the start of the reaction, and the amount of ADP-insensitive EP was measured by the kinetic method.

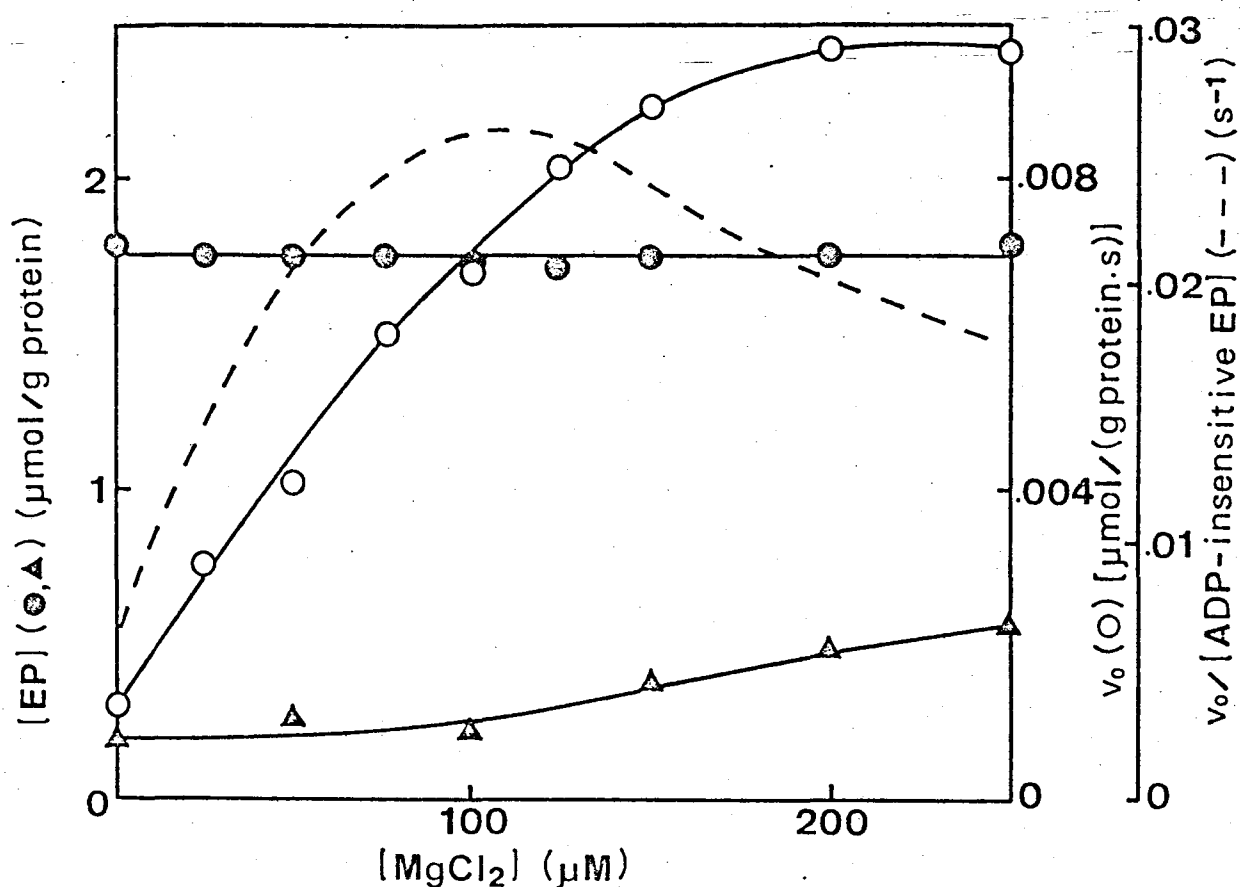


Fig. 16. Dependence of the v_0 value, $[\text{EP}]$ and the amount of ADP-insensitive EP on MgCl_2 concentration. The reaction was started by adding 1 μM AT^{32}P to SSR (0.3 mg/ml) in 0.1 M KCl, 0.1 M sucrose, 0.1 mM CaCl_2 , various concentrations of MgCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C, then the reaction was stopped 20 s later with 4% TCA, and the total amount of EP (\odot) and the value of v_0 (\circ) were measured. The amount of ADP-insensitive EP was measured by the simple method c.

unaffected by $[Mg^{2+}]$, as mentioned above. Thus, the value of $v_0/[EP]$ decreased slightly with an increase in $MgCl_2$ concentration (Fig. 15B). On the other hand, the amount of ADP-insensitive EP obtained by the kinetic method increased with an increase in $MgCl_2$ concentration, and about 90% of the total EP was ADP-insensitive in the presence of 5 mM $MgCl_2$.

Kanazawa et al. (11) reported that the decomposition of EP was accelerated by the addition of a low concentration of $MgCl_2$ (below 0.1 mM). Figure 16 shows the dependence of the ATPase activity, the total amount of EP, and the amount of ADP-insensitive EP at steady state on the concentration of added $MgCl_2$ in the range of 0 to 250 μM . The ATPase activity increased with an increase in the concentration of added $MgCl_2$. It was about 0.0012 $\mu mol/(g \text{ protein} \cdot s)$ when no $MgCl_2$ was added, and reached the maximum value, 0.0096 $\mu mol/(g \text{ protein} \cdot s)$, at $MgCl_2$ higher than 200 μM . The total amount of EP at steady state was constant over the whole range of $MgCl_2$ concentrations tested. The amount of ADP-insensitive EP increased from 0.18 to 0.56 $\mu mol/g \text{ protein}$ as the $MgCl_2$ increased from 0 to 250 μM . Therefore, the apparent rate constant of the decomposition of ADP-insensitive EP increased with an increase in the $MgCl_2$ concentration, and reached the maximum value (0.026 s^{-1}) at 0.1 mM $MgCl_2$, then decreased with a further increase of $MgCl_2$, as already shown in Fig. 15B.

Rate of Conversion of ADP-Sensitive EP into ADP-Insensitive EP — The formation of $AT^{32}P$ from $E^{32}P$ by addition of ATP + ADP showed a biphasic time course, and it was rather troublesome to determine the $AT^{32}P$ formed in the fast phase by extrapolating the slow phase to zero time. Therefore, I devised the simple method c.

The rationale for this method is given in Fig. 17. SSR (0.35 mg/ml) was phosphorylated with $1\ \mu\text{M}$ AT^{32}P in $1\ \text{mM}$ MgCl_2 and $40\ \mu\text{M}$ CaCl_2 , then $1\ \text{mM}$ ATP + $1\ \text{mM}$ ADP with or without $2\ \text{mM}$ EGTA was added 20 s later. The time courses of the decrease of E^{32}P , $^{32}\text{P}_i$ liberation, and AT^{32}P formation were measured. The formation of AT^{32}P after addition of ATP + ADP (+) showed a biphasic time course, while the amount of AT^{32}P formed after the addition of ATP + ADP + EGTA (X) did not change with time and the amount could be easily estimated by extrapolating to zero time. The amount of ADP-sensitive EP obtained by the simple method c, i.e., by adding ATP + ADP + EGTA, was usually 20% less than that obtained by the kinetic method. It should be added that the rate of $^{32}\text{P}_i$ liberation after the addition of ATP + ADP + EGTA or ATP + ADP was several times that at steady state ($0.018\ \mu\text{mol/g protein}\cdot\text{s}$). This indicates that high concentrations of ATP accelerate the decomposition of ADP-insensitive EP.

Figure 18 shows the time course of formation of ADP-insensitive EP from ADP-sensitive EP by removal of Ca^{2+} with EGTA. SSR was phosphorylated with $1\ \mu\text{M}$ AT^{32}P in $1\ \text{mM}$ MgCl_2 and $1\ \text{mM}$ CaCl_2 , then $5\ \text{mM}$ EGTA was added to stop the phosphorylation reaction 20 s later. After appropriate intervals, 4% TCA or $1\ \text{mM}$ ATP + $1\ \text{mM}$ ADP was added, and the amounts of total E^{32}P , $^{32}\text{P}_i$ liberated, and ADP-insensitive EP were measured. The amount of total E^{32}P decreased from 1.1 to $0.73\ \mu\text{mol/g protein}$ 5 s after addition of EGTA. The amount of $^{32}\text{P}_i$ liberated during 5 s ($0.22\ \mu\text{mol/g protein}$) was less than the decrease in E^{32}P . This indicates that ADP derived by ATP hydrolysis reacted with E^{32}P to form AT^{32}P . On the other hand, the amount of ADP-insensitive EP increased rapidly

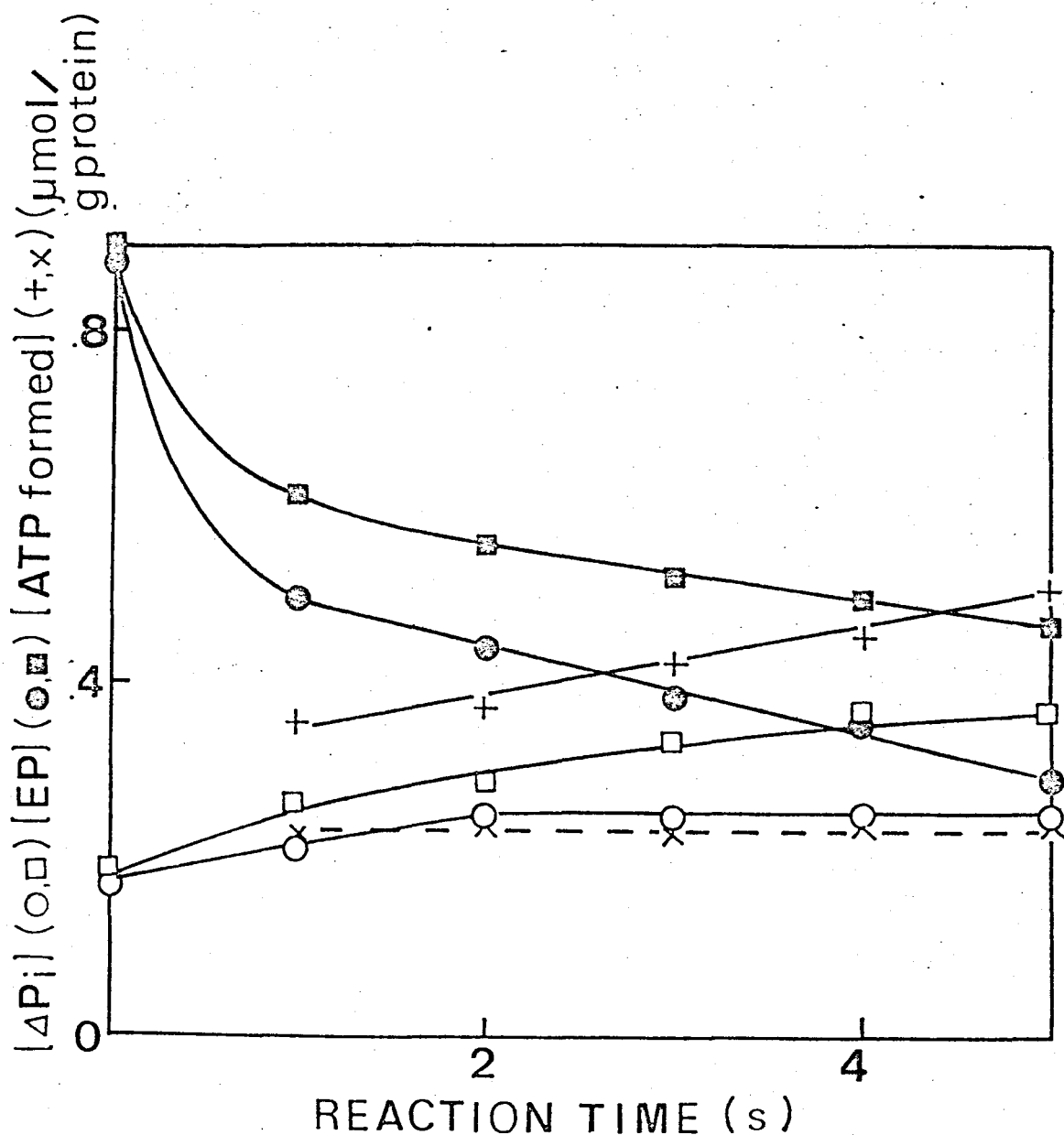


Fig. 17. Time courses of the decrease in $[E^{32}P]$, $^{32}P_i$ liberation and $ATP^{32}P$ formation caused by the addition of 1 mM unlabeled ATP + 1 mM ADP with or without 2 mM EGTA. SSR (0.35 mg/ml) was phosphorylated with 1 μM $AT^{32}P$ in the presence of 0.15 M KCl, 0.1 M sucrose, 1 mM $MgCl_2$, 40 μM $CaCl_2$, 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C, then 20 s later, 1 mM ATP + 1 mM ADP (○, ●, +) or 1 mM ATP + 1 mM ADP + 2 mM EGTA (□, ■, ×) was added. After appropriate intervals, the amounts of $^{32}P_i$ (○, □), $E^{32}P$ (●, ■) and $AT^{32}P$ (+, ×) were measured.

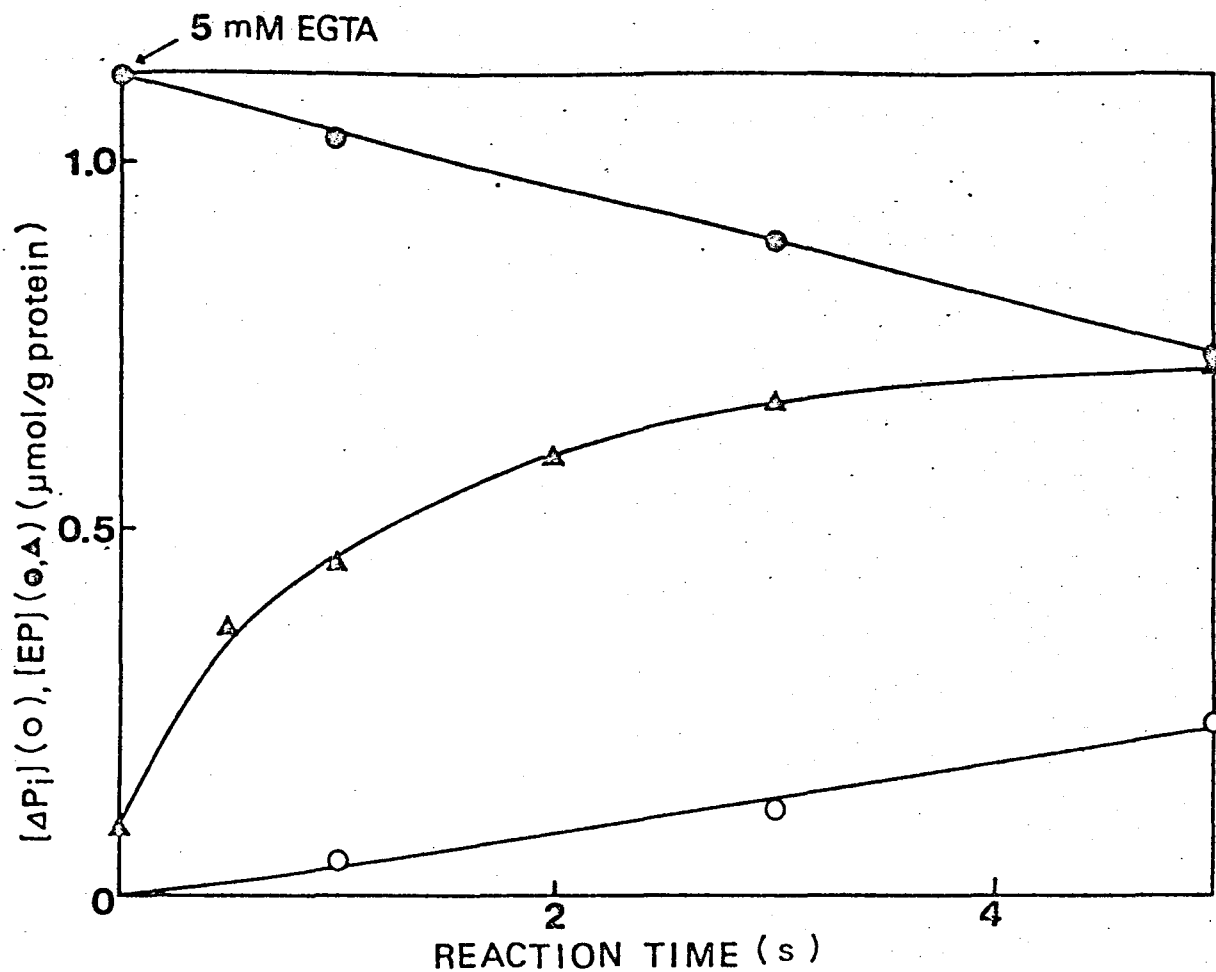
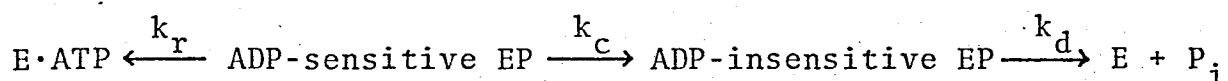


Fig. 18. Time course of formation of ADP-insensitive EP from ADP-sensitive EP by removal of Ca^{2+} ions with EGTA. SSR (0.4 mg/ml) was phosphorylated with $1 \mu\text{M}$ AT^{32}P in the presence of 0.1 M KCl, 0.1 M sucrose, 1 mM MgCl_2 , 1 mM CaCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C , then 5 mM EGTA was added 20 s later. After appropriate intervals, the amounts of P_i liberated (○), total EP (●) and ADP-insensitive EP (Δ) were measured.

after addition of EGTA. I assumed the following reaction scheme for the conversion of ADP-sensitive EP to ADP-insensitive EP:



The sum of $k_c + k_r$ was obtained as the first-order rate constant of the decrease of ADP-sensitive EP. I tentatively assumed that the value of k_r was equal to the first-order rate constant of total EP decomposition, and obtained 0.5 s^{-1} as the k_c value.

Figure 19 shows that the conversion of ADP-sensitive EP into -insensitive EP by removal of Ca^{2+} was unaffected by the addition of MgCl_2 . SSR was phosphorylated in the presence of various concentrations of MgCl_2 . The phosphorylation reaction was stopped by addition of 5 mM EGTA, and the time course of the formation of ADP-insensitive EP was measured. The amount of ADP-insensitive EP at zero time increased with an increase in MgCl_2 concentration, as mentioned above, while the rate of the conversion was almost constant over the entire concentration range tested.

Rate of Conversion of ADP-Sensitive EP into an Enzyme-ATP Complex — In the experiments shown in Fig. 20, SSR was phosphorylated with $1 \mu\text{M}$ AT^{32}P in 1 mM MgCl_2 and 1 mM CaCl_2 , where almost all the EP formed was ADP-sensitive. Twenty seconds later, the formation of E^{32}P was stopped by addition of 0.1 mM unlabeled ATP with various concentrations of ADP. The amount of E^{32}P decreased with the addition of ATP + ADP, while only a small amount of $^{32}\text{P}_i$ was liberated within 2 s after the addition of ATP + ADP (data not shown). Thus, the amount of decrease in E^{32}P was almost equal to the amount of AT^{32}P formed. The decrease in E^{32}P observed with addition of unlabeled ATP alone was

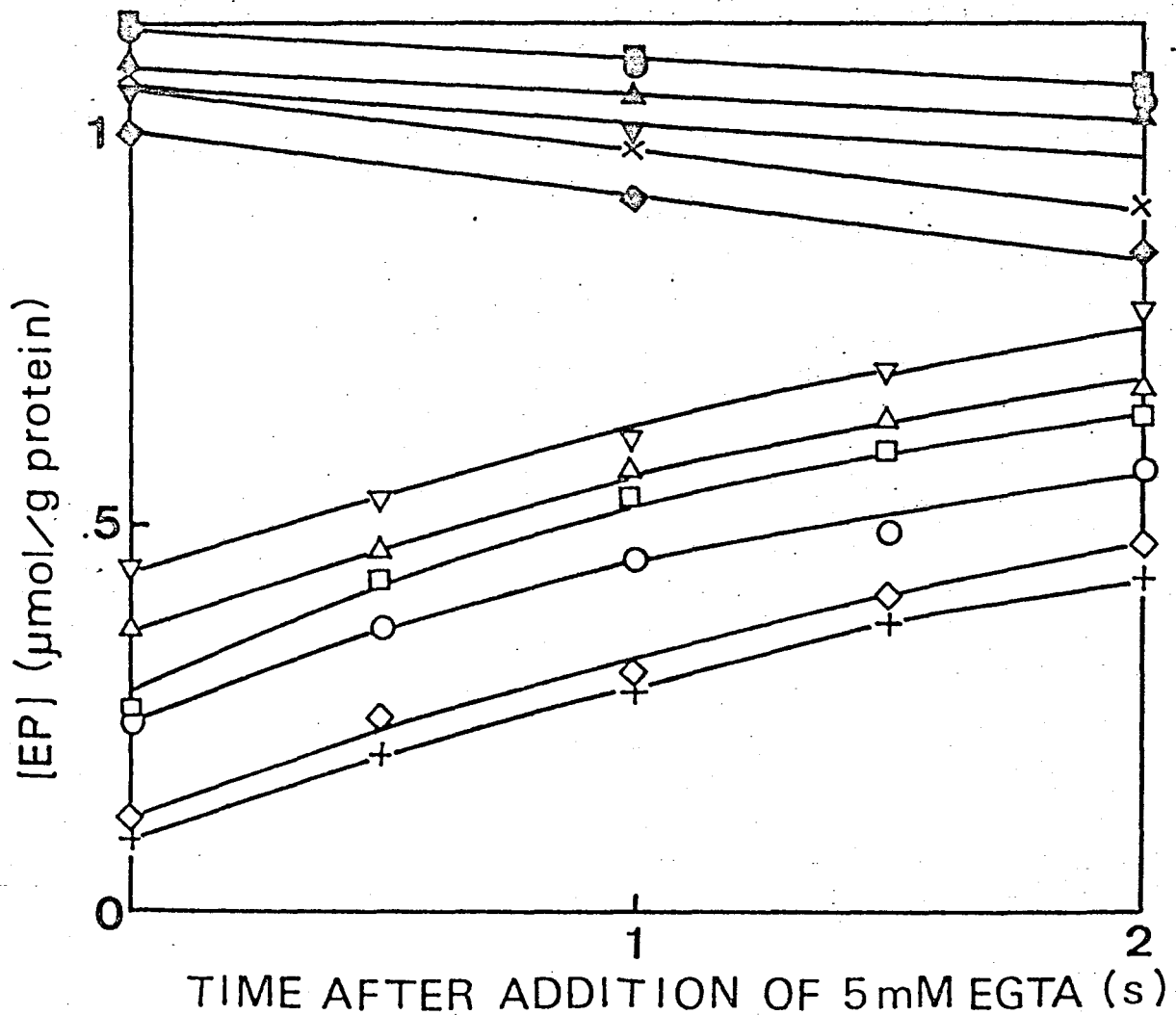


Fig. 19. Effect of MgCl_2 concentration on the rate of conversion of ADP-sensitive EP into ADP-insensitive EP. SSR (0.35 mg/ml) was phosphorylated with $1 \mu\text{M}$ AT^{32}P under the same conditions described for Fig. 18, except that the concentrations of MgCl_2 were 0 (\diamond, \blacklozenge), 0.1 ($+, \times$), 2 (\circ, \bullet), 3 (\square, \blacksquare), 5 ($\triangle, \blacktriangle$), and 10 ($\nabla, \blacktriangledown$) mM. The reaction was stopped with 5 mM EGTA 20 s after its start, and the amounts of total EP ($\blacklozenge, \times, \bullet, \blacksquare, \blacktriangle, \blacktriangledown$) and ADP-insensitive EP ($\diamond, +, \circ, \square, \triangle, \nabla$) were measured.

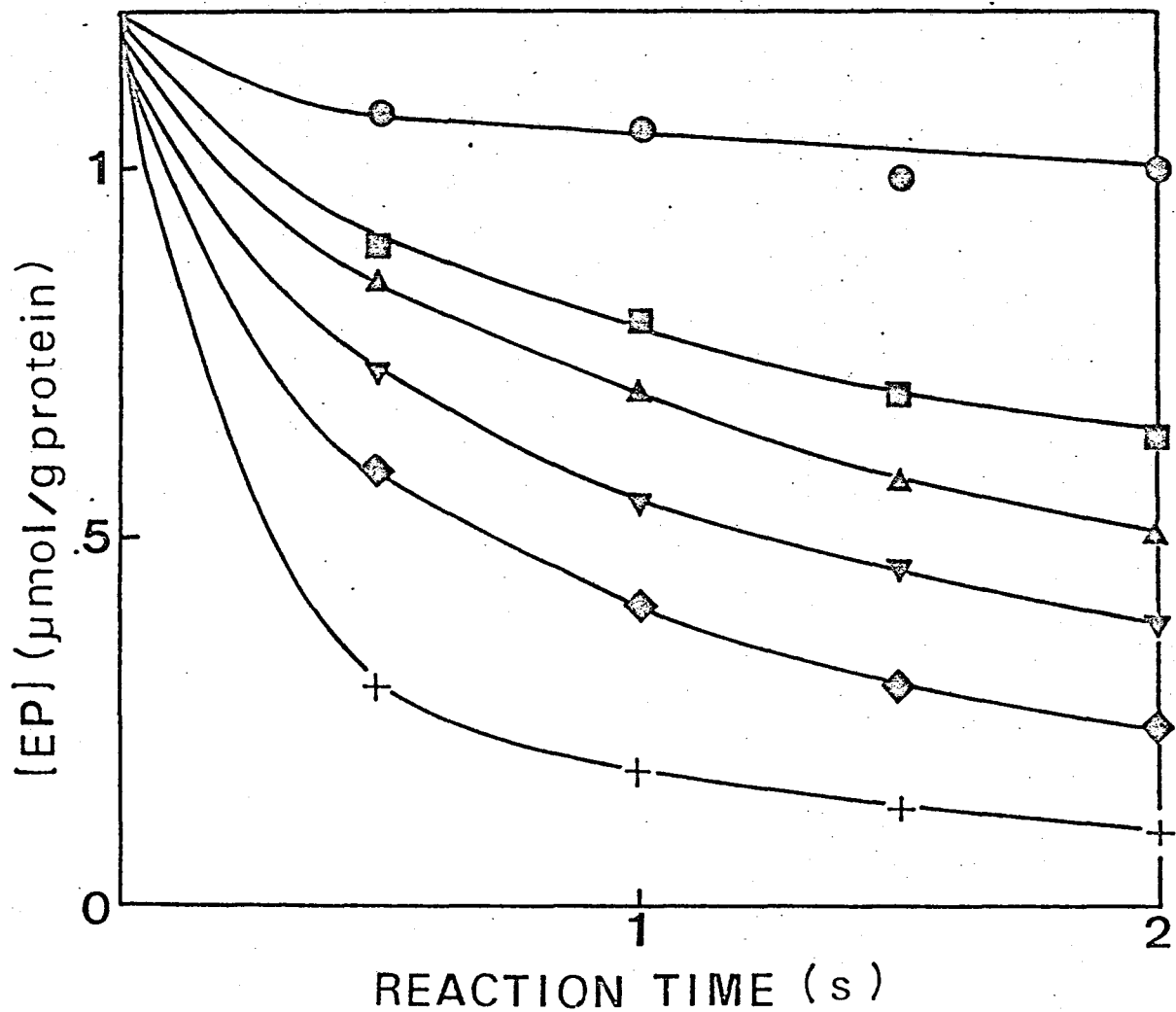


Fig. 20. Time course of decrease in the amount of ADP-sensitive EP by the reaction with ADP. SSR (0.4 mg/ml) was phosphorylated with 1 μ M AT^{32}P in the presence of 0.1 M KCl, 0.1 M sucrose, 1 mM MgCl_2 , 1 mM CaCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C. The formation of E^{32}P was stopped by adding 0.1 mM unlabeled ATP with 0 (●), 10 (■), 15 (▲), 20 (▼), 40 (◆), and 100 (×) μ M ADP.

attributed to the contamination of unlabeled ATP preparation with a small amount of ADP. The rate of AT^{32}P formation increased with increase in the concentration of ADP added together with ATP. The time course of AT^{32}P formation did not follow simple first-order kinetics, indicating the existence of at least two kinds of ADP-sensitive EP; one could react with ADP rapidly and the other rather slowly.

Rate of Conversion of ADP-Insensitive EP into ADP-Sensitive EP — In the experiments shown in Fig. 21, SSR was phosphorylated with $1\ \mu\text{M}$ AT^{32}P in $1\ \text{mM}$ MgCl_2 and $10\ \mu\text{M}$ CaCl_2 , where almost all the EP was ADP-insensitive. Twenty seconds later, various concentrations of CaCl_2 were added, and the time course of conversion of ADP-insensitive EP into -sensitive EP was measured. The amount of ADP-insensitive EP after addition of CaCl_2 was represented as the percent of the amount just before the addition of CaCl_2 . The time course of decrease in the amount of ADP-insensitive EP after adding CaCl_2 consisted of fast and slow phases. When a high concentration of CaCl_2 was added to the reaction mixture, the total amount of EP decreased very slowly, and its rate was almost equal to that of the slow phase of decrease in ADP-insensitive EP. Therefore, it was concluded that the slow phase was due to a decrease in the total amount of EP by the reaction of EP with a small amount of ADP derived from AT^{32}P hydrolysis to form an enzyme-ATP complex.

Inhibition of Conversion of ADP-Sensitive EP into ADP-Insensitive EP by Modification with MalNet — Figure 22 shows the time courses of changes in the ATPase activity, the total amount of EP, and the amount of ADP-insensitive EP, when SSR ($1.5\ \text{mg/ml}$)

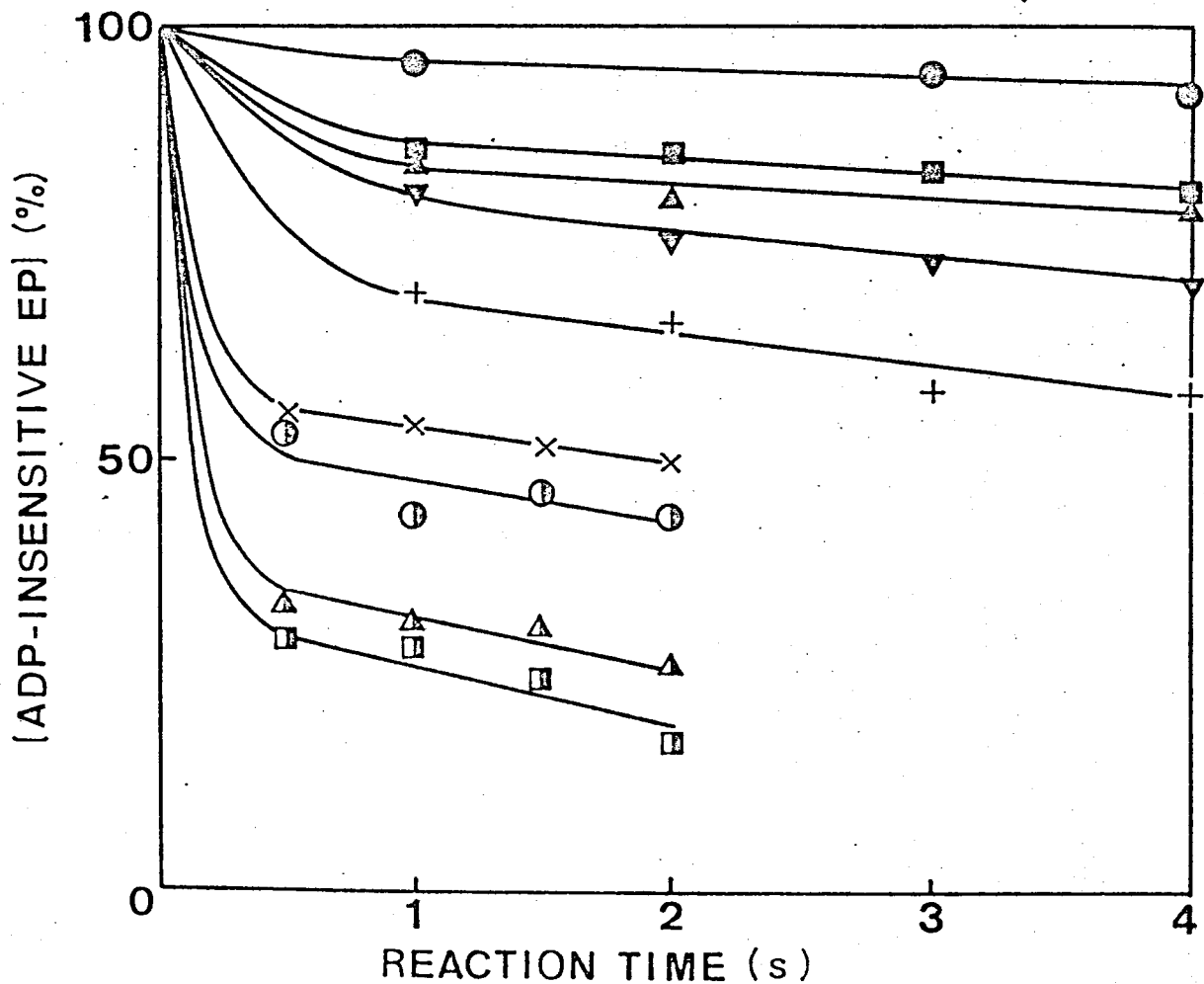


Fig. 21. Time course of conversion of ADP-insensitive EP into ADP-sensitive EP by addition of CaCl_2 . SSR (0.6 mg/ml) was phosphorylated with $1 \mu\text{M}$ AT^{32}P in the presence of 0.1 M KCl, 0.1 M sucrose, 1 mM MgCl_2 , $10 \mu\text{M}$ CaCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C . The time courses of the decrease in ADP-insensitive EP due to addition of 30 (●), 60 (■), 90 (▲), 120 (▼), 200 (+), 500 (×), 1000 (⊙), 2000 (⊴), and 5000 (⊞) μM CaCl_2 were measured.

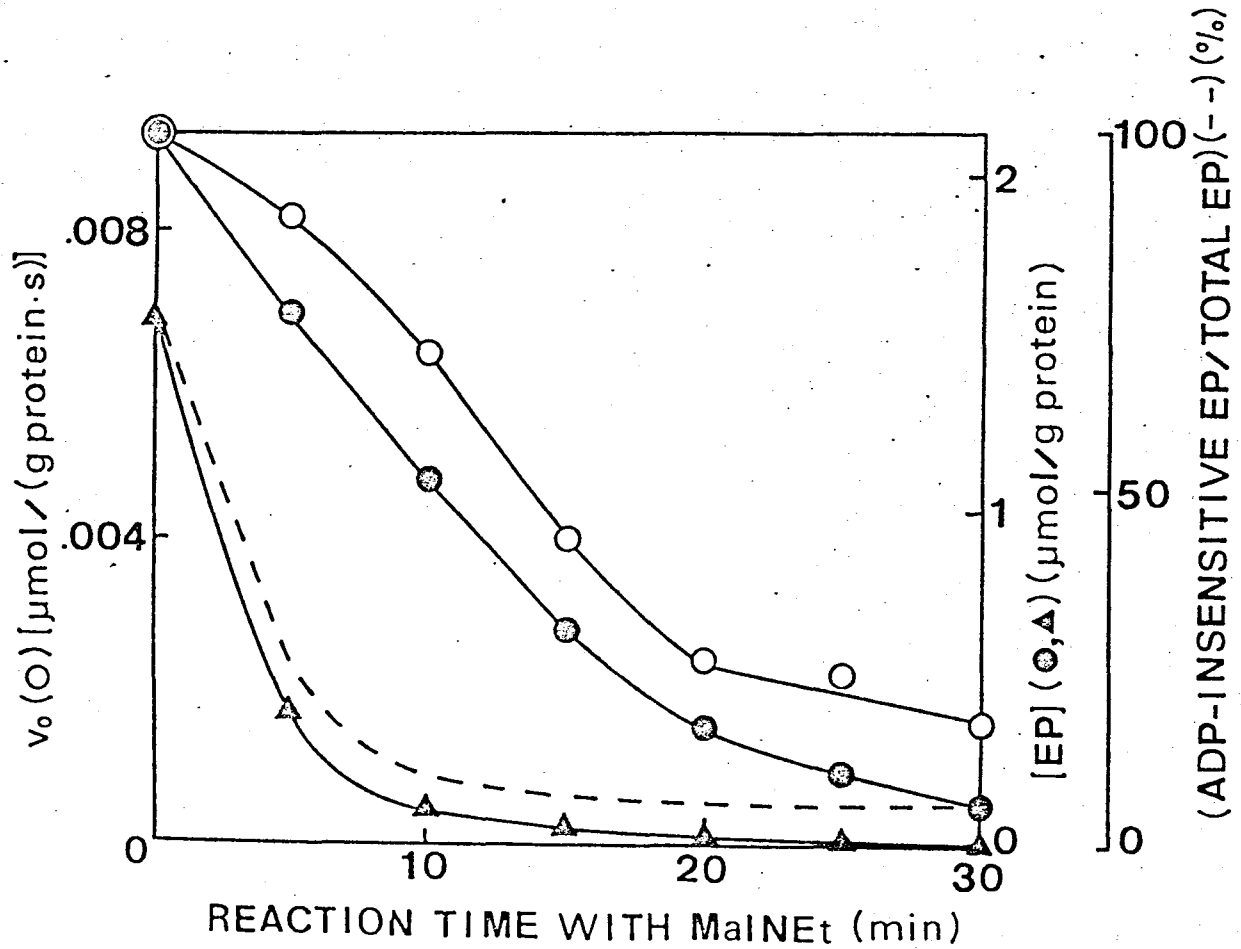


Fig. 22. Time courses of changes in the ATPase activity, the total amount of EP, and the amount of ADP-insensitive EP by the reaction of SSR with MalNEt. SSR (1.5 mg/ml) was reacted with 1 mM MalNEt in the presence of 0.35 M KCl, 0.25 M sucrose, 0.1 mM CaCl_2 , 0.1 mg/ml Tween 80, and 30 mM Tris-HCl at pH 7.8 and 20°C, and the reaction was stopped with 10 mM DTT. MalNEt-treated SSR (0.3 mg/ml) was phosphorylated with 1 μM AT^{32}P in the presence of 0.1 M KCl, 0.1 M sucrose, 10 mM MgCl_2 , 0.1 mM CaCl_2 , 0.1 mg/ml Tween 80 and 100 mM Tris-HCl at pH 9.0 and 10°C, and the v_o value (O), the amounts of total EP (●) and ADP-insensitive EP (Δ) were measured.

reacted with 1 mM MalNet, as described in "EXPERIMENTAL PROCEDURE." The reaction with MalNet was stopped by addition of 10 mM DTT, and the ATPase activity and the total amount of EP were measured in the presence of 10 mM MgCl_2 and 0.1 mM CaCl_2 . The amount of ADP-insensitive EP was measured by the simple method c. The ATPase activity, the total amount of EP, and the amount of ADP-insensitive EP decreased with the time of the MalNet treatment. When the ratio of ADP-insensitive EP to total EP was plotted against the incubation time with MalNet, the ratio decreased from 75% to below 10% by the reaction with MalNet for 10 min.

Figure 23 shows the rate of conversion of ADP-sensitive EP into -insensitive EP by removal of Ca^{2+} using SSR treated with MalNet for 10 min. The modified SSR (0.38 mg/ml) was phosphorylated with 1 μM AT^{32}P in the presence of 1 mM MgCl_2 and 1 mM CaCl_2 , and the rate of conversion was measured as described in Fig. 18. The total amount of EP decreased slowly from 0.91 to 0.58 $\mu\text{mol/g}$ protein in 5 s when 5 mM EGTA was added. The amount of P_i liberated during 5 s was 0.04 $\mu\text{mol/g}$ protein. Thus, the reverse reaction of EP formation occurred on addition of EGTA. The amount of ADP-insensitive EP increased very slowly on addition of EGTA, and the rate constant of conversion of ADP-sensitive EP into -insensitive EP calculated from the results shown in Fig. 23 was about 0.1 s^{-1} , which was about fivefold smaller than that of intact SSR, 0.5 s^{-1} (cf. Fig. 18).

The inhibition of the conversion by MalNet treatment is also shown in Fig. 24. SSR treated with MalNet for 10 min (0.38 mg/ml) was phosphorylated with 1 μM AT^{32}P in the presence of various concentrations of MgCl_2 and 0.1 mM CaCl_2 , and the total amount of

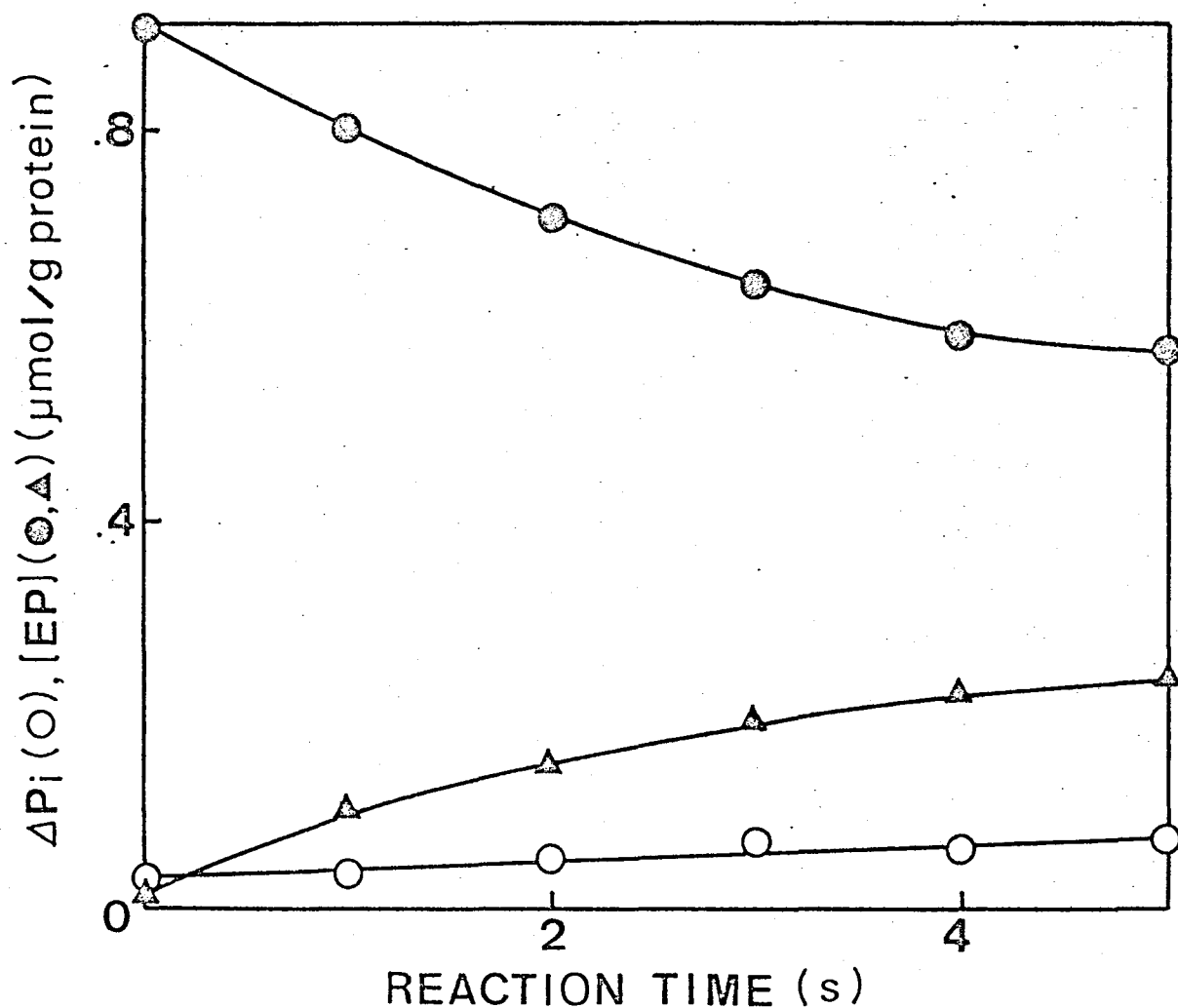


Fig. 23. Inhibition of conversion of ADP-sensitive EP into ADP-insensitive EP by modification of ATPase with MalNEt. SSR treated with MalNEt for 10 min (0.38 mg/ml) was phosphorylated with $1 \mu\text{M}$ AT^{32}P in the presence of 0.1 M KCl, 0.1 M sucrose, 1 mM MgCl_2 , 1 mM CaCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C , then 5 mM EGTA was added 20 s later. After appropriate intervals, the amounts of P_i liberated (O), total EP (●), and ADP-insensitive EP (▲) were measured.

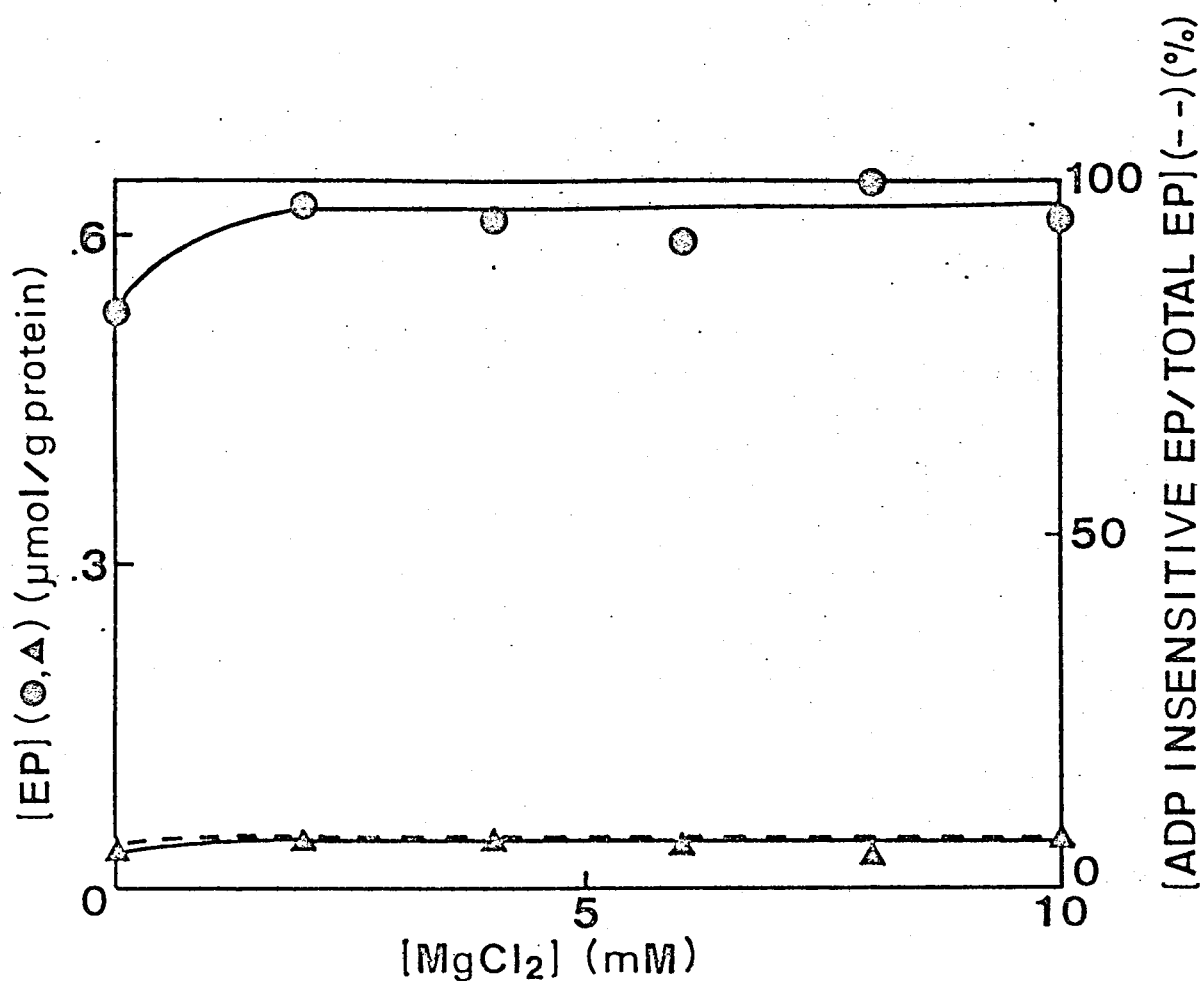


Fig. 24. Effect of MgCl_2 concentration on the conversion of ADP-sensitive EP into ADP-insensitive EP of MalNet-treated SSR. SSR treated with 1 mM MalNet for 10 min (0.58 mg/ml) was phosphorylated with 1 μM AT^{32}P in the presence of 0.1 M KCl, 0.1 M sucrose, various concentrations of MgCl_2 , 0.1 mM CaCl_2 , 0.1 mg/ml Tween 80, and 100 mM Tris-HCl at pH 9.0 and 10°C. The formation of E^{32}P was stopped with 4% TCA or 1 mM ATP + 1 mM ADP + 2 mM EGTA, and the amounts of total EP (●) and ADP-insensitive EP (Δ) were measured.

EP and the amount of ADP-insensitive EP were measured 20 s after the reaction had been started. The amount of total EP and that of ADP-insensitive EP were almost constant over the whole range of MgCl_2 concentrations tested. The ratio of ADP-insensitive EP to total EP was less than 10% which was much smaller than the value of intact SSR, i.e., 75% in the presence of 10 mM MgCl_2 (cf. Fig. 13B).

DISCUSSION

When FSR was solubilized with detergent, neither the EP overshoot nor the P_i burst in the initial phase of the reaction were observed, and the ATPase reaction could be easily analyzed (6,12) (see Part I). SSR ATPase used in this study also showed neither the EP overshoot nor the P_i burst. The dependence on ATP concentration of the rate of the SSR ATPase reaction and the amount of EP in the steady state as well as the initial velocity of EP formation followed the simple Michaelis-Menten equation (Fig. 3). The maximum amount of EP formed was about $2 \text{ mol}/10^6 \text{ g protein}$, and was considerably less than $10 \text{ mol}/10^6 \text{ g protein}$ which was obtained by assuming the molecular weight per ATPase active site to be 10^5 g (13-15). There are two explanations for the small amount of EP. One is that only 20% of our SSR ATPase preparation was active. The other is that the maximum concentration of EP is smaller than the active site concentration, because the enzyme assumes a new state after EP decomposition, which cannot bind ATP, and the rate of conversion from this state to the original one is of the same order of magnitude as the rate of EP decomposition. However, the

second mechanism seems unlikely, because EP overshoot was not observed in the initial phase of the reaction.

The first interesting finding in this study obtained with SSR ATPase, where the problem of the production of Ca^{2+} gradient during the ATPase reaction could be avoided, was that ADP-sensitive EP is a true reaction intermediate. Thus, I found that the ATPase activity and the amount of EP at steady state were non-competitively inhibited by ADP and the rate constant of EP decomposition ($v_0/[\text{EP}]$) was constant over a wide range of ADP concentrations (Fig. 5). Furthermore, the reaction of EP with ADP was measured both in the presence and absence of CaCl_2 at alkaline pH where EP decomposed very slowly, and two kinds of EP (ADP-sensitive and -insensitive EP) were found in the presence of sufficient amounts of added alkali metal salts (see Fig. 6 & 7). The existence of the two kinds of EP was previously reported for purified ATPase by Shigekawa et al. (16-18), who performed the experiments in the absence of added alkali metal salts. They also reported that all the EP formed in the presence of a sufficient amount of alkali metal salts was ADP-sensitive (see INTRODUCTION). Kanazawa et al. (11) also reported that the extent of AT^{32}P formation from E^{32}P of FSR and added ADP decreased gradually with time after the addition of EGTA, and that ATP formation from EP and ADP was instantaneously inhibited by the addition of EGTA when FSR was pre-treated with Triton X-100. Thus, they concluded that Ca^{2+} ions inside the vesicle are essential for the AT^{32}P formation from E^{32}P and ADP. Therefore, the discrepancy between Shigekawa's and my results may be due to the difference of ATPase preparations used: Shigekawa et al. used purified ATPase which retained the membrane structure,

while I used solubilized ATPase.

The second finding was that the two kinds of EP are formed sequentially: ADP-sensitive EP is formed first, then converted into ADP-insensitive EP (see Figs. 10 & 11). Shigekawa and Dougherty (18) also reported the sequential formation of two kinds of EP. The rate of conversion of ADP-sensitive EP into -insensitive EP was faster than the rate of decomposition of ADP-insensitive EP. For example, at pH 9.0 and 10°C, the rate of conversion was about 0.5 s^{-1} , while the rate of decomposition was less than 0.01 s^{-1} (Fig. 11). However, the amount of ADP-sensitive EP was larger than that of ADP-insensitive EP under these conditions. Therefore, I concluded that ADP-sensitive and -insensitive EP are in equilibrium. In spite of rapid conversion between ADP-sensitive and -insensitive EP, they could be distinguished by the difference in their reactivities with ADP. These results indicated that the rate of conversion of ADP-insensitive EP into -sensitive EP with bound ADP was considerably slower than the rate in the absence of ADP. This conclusion was supported by the finding that the time courses of AT^{32}P formation from E^{32}P and ADP consisted of fast and slow phases (Figs. 14A and 15A).

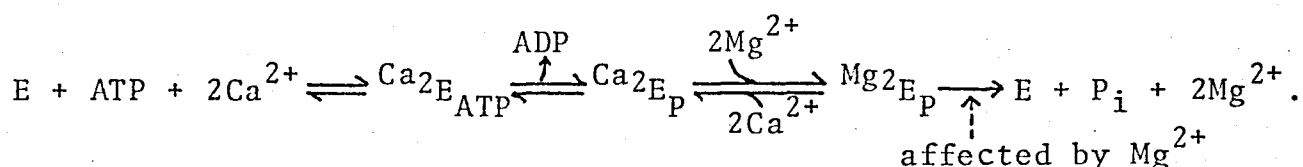
The third finding was that the ratio of the two kinds of EP is dependent on the concentrations of MgCl_2 and CaCl_2 , as previously reported by Shigekawa and Dougherty (18) for purified ATPase in the absence of added alkali metal salts. As shown in Fig. 13, the dependences were given by

$$\frac{[\text{ADP-insensitive EP}]}{[\text{total EP}]} = \frac{1}{1 + \left\{ \frac{K_{\text{Mg}} [\text{Ca}^{2+}]}{K_{\text{Ca}} [\text{Mg}^{2+}]} \right\}^2}$$

This indicates that ADP-sensitive EP binds two moles of Ca^{2+} and ADP-insensitive EP binds two moles of Mg^{2+} per mole of EP. This result was consistent with the mechanism that two moles of Ca^{2+} are transported coupled with a mole of ATP-hydrolysis (13-15). The value of $K_{\text{Mg}}/K_{\text{Ca}}$ for EP was about 50 (see Fig. 13), which was 600 times smaller than that of $K_{\text{Mg}}/K_{\text{Ca}}$ for E obtained by Yamada and Tonomura (19). Thus, the affinities of the enzyme for Ca^{2+} and Mg^{2+} change dramatically with the EP formation.

Since the ADP-insensitive EP is considered to be the terminal intermediate, the effects of Ca^{2+} and Mg^{2+} on its decomposition were measured. It was unaffected by CaCl_2 (Fig. 14) and seemed to be accelerated by low concentrations of MgCl_2 (Fig. 16), but it was inhibited by a high concentration of MgCl_2 (Fig. 17).

All these results could be easily explained by assuming two forms (Ca and Mg forms) of EP, which are formed sequentially:



The existence of two forms of SSR ATPase has directly been shown by Nakamura and Tonomura (20) from the change in the UV spectrum of SSR ATPase induced by Ca^{2+} removal.

The time course of the conversion between ADP-sensitive and -insensitive EP and that of the formation of E·ATP complex from ADP-sensitive EP and ADP could be explained, assuming the existence of two conformational states both in ADP-sensitive and -insensitive EP:



As shown in Fig. 16, the conversion of ADP-sensitive EP into -insensitive EP followed first-order kinetics. However, the rate constant was independent of $[Mg^{2+}]$ (Fig. 17). Therefore, the increase in ADP-insensitive EP due to the addition of Mg^{2+} was concluded to be caused by the reaction of Mg^{2+} with ADP-insensitive $*E_p$ to form ADP-insensitive E_p . The biphasic time course of conversion of ADP-insensitive EP into -sensitive EP by addition of Ca^{2+} also supported the above conclusion. Furthermore, the biphasic time course of conversion of ADP-sensitive EP into $E \cdot ATP$ complex indicated the existence of two different conformational states of ADP-sensitive EP.

The fourth finding was that the conversion of ADP-sensitive EP into -insensitive EP is inhibited by the modification of ATPase with MalNet. It is well known that in the reaction of Na^+ , K^+ -dependent ATPase, ADP-sensitive and -insensitive EP are formed sequentially (21), and that the conversion of ADP-sensitive EP into -insensitive EP is inhibited by the modification of ATPase with MalNet (22). Thus, the kinetic properties of the two kinds of EP in the reaction of Ca^{2+} , Mg^{2+} -dependent ATPase are very similar to those of Na^+ , K^+ -dependent ATPase. However, Kanazawa et al. (7) and Fukushima and Tonomura (23) found the existence of E_p^{ADP} and Yamaguchi and Tonomura (24) found the existence of E^{ADP} intermediates in the reaction of Na^+ , K^+ -dependent ATPase, and Hexum et al. (25) found that the mode of ADP inhibition of Na^+ , K^+ -dependent ATPase activity is the competitive type. On the other hand, I found that EP intermediates of Ca^{2+} , Mg^{2+} -dependent ATPase of SR do not contain bound ADP (6), and that SR ATPase is non-competitively inhibited by ADP (cf. Fig. 5). Thus, these

results strongly suggest that the main route of the Na^+ , K^+ -dependent ATPase reaction is the decomposition of E_p^{ADP} into $\text{E}^{\text{ADP}} + \text{P}_i$, while that of Ca^{2+} , Mg^{2+} -dependent ATPase is the decomposition of E_p^{ADP} into $\text{E}_p + \text{ADP}$.

* * * * *

A part of this investigation will be published in Cation Flux Across Biomembranes (Mukohata, Y., and Packer, L., eds.), Academic Press, San Francisco.

REFERENCES

1. Yamada, S., Yamamoto, T., & Tonomura, Y. (1970) J. Biochem. 67, 789-794
2. le Maire, M., Møller, J.V., & Tanford, C. (1976) Biochemistry 15, 2336-2342
3. Weber, K. & Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412
4. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468
5. Glynn, I.M. & Chappell, J.B. (1964) Biochem. J. 90, 965-967
6. Takisawa, H. & Tonomura, Y. (1978) J. Biochem. 83, 1275-1284
7. Kanazawa, T., Saito, M., & Tonomura, Y. (1970) J. Biochem. 67, 693-711
8. Wadkins, C.L. & Lehninger, A.L. (1963) in Methods in Enzymology (Colowick, S.P. & Kaplan, N.O. eds.) Vol. 6, p 268, Academic Press, New York
9. Cleland, W.W. (1970) in The Enzymes (Boyer, P.D. ed.) Vol. 2, pp. 1-65, Academic Press, New York
10. Yamamoto, T. & Tonomura, Y. (1967) J. Biochem. 64, 137-145
11. Kanazawa, T., Yamada, S., Yamamoto, T., & Tonomura, Y. (1971) J. Biochem. 70, 95-123
12. Yamada, S., Yamamoto, T., Kanazawa, T., & Tonomura, Y. (1971) J. Biochem. 70, 279-291
13. Tonomura, Y. (1972) Muscle Proteins, Muscle Contraction and Cation Transport, Univ. Tokyo Press & Univ. Park Press, Tokyo & Baltimore
14. Tada, M., Yamamoto, T., & Tonomura, Y. (1978) Physiol. Rev. 58, 1-79

15. Yamamoto, T., Takisawa, H., & Tonomura, Y. (1979) in Current Topics in Bioenergetics (Sanadi, R. ed.) Vol. 9, Academic Press, New York in press
16. Shigekawa, M., Dougherty, J.P., & Katz, A.M. (1978) J. Biol. Chem. 253, 1442-1450
17. Shigekawa, M. & Dougherty, J.P. (1978) J. Biol. Chem. 253, 1451-1457
18. Shigekawa, M. & Dougherty, J.P. (1978) J. Biol. Chem. 253, 1458-1464
19. Yamada, S. & Tonomura, Y. (1972) J. Biochem. 72, 417-425
20. Nakamura, Y. & Tonomura, Y. (1979) submitted to J. Biochem.
21. Post, R.L., Kume, S., Tobin, T., Orcutt, B., & Sen, A.K. (1969) J. Gen. Physiol. 54, 306s-321s
22. Fahn, S., Koval, G.J., & Albers, R.W. (1966) J. Biol. Chem. 241, 1882-1888
23. Fukushima, Y. & Tonomura, Y. (1974) Ann. N. Y. Acad. Sci. 242, 92-105
24. Yamaguchi, M. & Tonomura, Y. (1978) J. Biochem. 83, 977-987
25. Hexum, T., Sanson, F.E. Jr., & Himes, R.H. (1970) Biochim. Biophys. Acta 212, 322-331

SUMMARY

Solubilized Ca^{2+} , Mg^{2+} -dependent ATPase with stable activity was prepared from sarcoplasmic reticulum of skeletal muscle by a modified method of le Maire et al. (1976) (Biochemistry 15, 2336-2342). The dependence on ATP concentration of the ATPase activity (v_0) and the amount of EP in the steady state and the initial rate of EP formation followed the Michaelis-Menten equation.

ADP inhibited the ATPase activity non-competitively and the value of $v_0/[\text{EP}]$ was constant over a wide range of ADP concentrations. These results indicated the existence of EP which decreased with the addition of ADP. The amount of two kinds of EP, one of which could react with ADP to form ATP and the other which could not, were measured at alkaline pH where the EP decomposition was very slow. The following results were obtained.

1. In the presence of a sufficient amount of Ca^{2+} , almost all the EP formed reacted with ADP to form ATP (ADP-sensitive), while in the absence of Ca^{2+} , all the EP formed could not react with ADP (ADP-insensitive).
2. When the reaction was started by adding ATP to the ATPase, ADP-sensitive EP was formed first, then converted into ADP-insensitive EP.
3. The dependence on Mg^{2+} and Ca^{2+} concentrations of the amounts of the two kinds of EP indicated that ADP-sensitive EP binds two mol of Ca^{2+} (Ca_2EP) and ADP-insensitive EP binds two mol of Mg^{2+} per mol of EP (Mg_2EP). Furthermore, the affinities of the enzyme for Ca^{2+} and Mg^{2+} changed markedly with the phosphorylation reaction.

4. The decomposition of ADP-insensitive EP was accelerated by the addition of a low concentration of Mg^{2+} and inhibited by a high concentration of Mg^{2+} . It was unaffected by Ca^{2+} ions.
5. The conversion of ADP-sensitive EP into -insensitive EP followed first-order kinetics ($k = 0.5 \text{ s}^{-1}$ at pH 9.0 and 10°C), and was unaffected by the addition of Mg^{2+} . Both the time course of the conversion of ADP-insensitive EP into -sensitive EP and that of the formation of E-ATP complex from ADP-sensitive EP and ADP were biphasic. These results indicated that both ADP-sensitive and -insensitive EP have at least two different conformational states.
6. The conversion of ADP-sensitive EP into -insensitive EP was inhibited by the modification of ATPase with MalNEt.

ACKNOWLEDGMENT

The author would like to express his great appreciation to Professor Yuji Tonomura for his kind guidance, many valuable suggestions, and continuous encouragement during the course of this work. Great thanks should be given to Drs. A. Inoue , T. Watanabe and all the members of Tonomura-Laboratory for their valuable discussions and to Dr. S. K. Srivastava for critically reading the manuscript. The author is greatly indebted to Drs. T. Yamamoto, M. Sumida and Mr. Y. Nakamura for their cooperation in experiments. Finally, the author would like to express his great thanks to Professor S. Watanabe (Tokyo Institute of Technology) , and also Associate Professor Y. Inada who was a guidance teacher during the course of my work on Master of Science, because their kind recommendation enabled me to start this work at Osaka University.