and evaluated. H is taken to be 4.80×10^{-5} cm^2/g^2 (Butler, 1972), while λ' is taken to be 228 nm (observations performed at 310-nm wavelength in air; refractive index of solution = 1.36). The value of $d\tau/dt$ is the initial rate of turbidity change, C_i and D_i are obtained from the electron microscopic determinations of rod numbers and lengths, while S_i and Q_i are taken from Doty and Steiner (1950).

Calculations from eq 10 of the rate of elongation of partially assembled rods give the *average* for all the rods in the PAR preparation (Table I). Only a fraction of the rods, however, will be capable of further growth, those rods having a pendant RNA "tail." The presence of tailless rods results in a calculated average growth rate that is less than the true one. We estimate from measurements of the length distribution of the PAR at $t = 0$, and after further growth has reached completion (*e.g.*, see Figures 3A and 3B), that the active rods comprise ~70% of the total number of PAR. The average rate of growth for those rods that are active is estimated to be about 80 nm/min.

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Phosphorylation of the Sarcoplasmic Reticulum Membrane by Orthophosphate. Inhibition by Calcium Ions†

Hatisaburo Masuda‡ and Leopoldo de Meis*

ABSTRACT: A protein of the sarcoplasmic reticulum membrane of skeletal muscle is phosphorylated by orthophosphate at pH 6.0 in the presence of Mg^{2+}. The orthophosphate concentration required for half-maximal phosphorylation is 1.06 mM. This reaction is strongly inhibited by Ca^{2+} and to a lesser extent by Na^+ and K^+. ATP and ADP compete with orthophosphate for the membrane phosphorylating sites, ATP being a more effective inhibitor than ADP. It is suggested that nucleoside triphosphates and orthophosphate interact with the same site on the membrane and that the binding of Ca^{2+} determines which of them will phosphorylate the membrane.

Fragmented SRV[1] isolated from skeletal muscle retain a highly efficient ATP-dependent Ca^{2+} transport system (Hasselbach and Makinose, 1961; Hasselbach, 1964). Makinose (1969) has shown that in the process of ATP hydrolysis, the γ-phosphate of ATP is covalently bound to a membrane protein (E). The phosphoprotein (E~P) represents an intermediate in the sequence of reactions leading to Ca^{2+} transport and phosphate liberation. Makinose (1969) has therefore proposed the following reaction sequence.

$$ATP + E \overset{Ca^{2+}}{\rightleftharpoons} E\sim P + ADP \quad (1)$$

$$E\sim P \longrightarrow E + P_i \quad (2)$$

Recently it has been shown that under specific conditions, the Ca^{2+} pump of the SRV can be reversed, *i.e.*, that reaction 2 shown above is reversible. When SRV previously loaded with calcium oxalate or calcium phosphate are incubated in a medium containing ADP, Mg^{2+}, and [^{32}P]P_i, it is observed that [^{32}P]P_i interacts with the membrane forming E~P (Makinose, 1972), Ca^{2+} is released at a very high rate (Barlogie *et al.*, 1971), and [γ-^{32}P$_i$]ATP is formed (Makinose, 1971; Makinose and Hasselbach, 1971). In these experiments it was

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[1] Abbreviations used are: SRV, sarcoplasmic reticulum vesicles; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

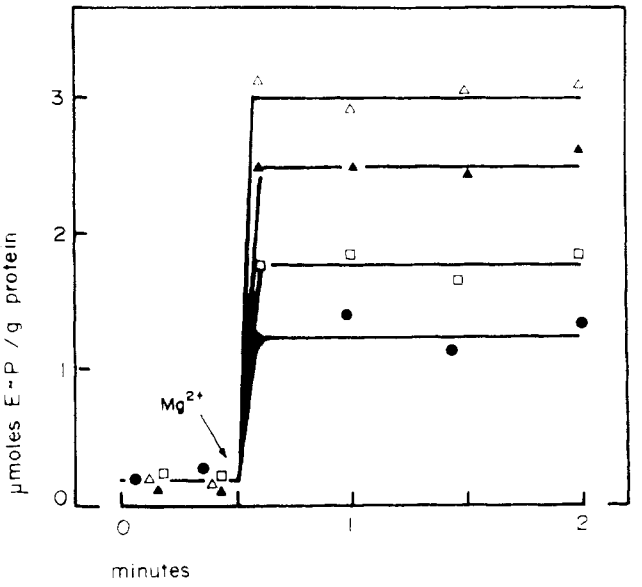

FIGURE 1: Time course of E∼P formation and its dependence on P_i concentration and Mg^{2+}. The assay medium composition was 10 mM Tris-maleate buffer (pH 6.0), 1 mM EGTA, and 0.5 mM (●), 1 mM (□), 2 mM (▲), or 4 mM (△) [^{32}P]P_i. The arrow points to the addition of $MgCl_2$ with a final concentration of 10 mM. Other additions and experimental conditions were as described under Materials and Methods.

shown that the energy required for E∼P formation and subsequent ATP synthesis was derived from the steep Ca^{2+} concentration gradient formed across the SRV membrane.

This paper reports that [^{32}P]P_i can react with the SRV forming a high energy E∼P bound in the absence of a Ca^{2+} concentration gradient.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as previously described (de Meis and Hasselbach, 1971).

Standard Assay. Unless otherwise stated, the incubation medium consisted of 10 mM Tris-maleate buffer (pH 6.0), 1 mM EGTA, 10 mM $MgCl_2$, and different concentrations of [^{32}P]P_i at pH 6.0. The final volume was 2.5 ml. The reaction was started by the addition of SRV, a total of 0.6 mg of protein/ml, and stopped after different incubation intervals at 37° by injecting the assay medium into 20 ml of an ice-cold solution of 125 mM perchloric acid containing 2 mM orthophosphate. The suspensions were centrifuged in the cold at 5000*g* for 15 min. The protein pellet was washed four times with 20-ml samples of ice-cold 125 mM perchloric acid solution containing 2 mM orthophosphate. After the washings, the pellet was resuspended in 1 ml of a solution containing 0.1 N NaOH, 2% Na_2CO_3, and 1 mM orthophosphate. The pellet was dissolved by heating the suspension in boiling water for 30 min. After cooling, an aliquot was dried in a planchet and counted in a Nuclear-Chicago gas flow counter. Another aliquot was used for protein determination by the method of Lowry *et al.* (1951).

In order to measure the unspecific binding of [^{32}P]P_i, control tests were performed in which the SRV was denaturated by 20 mM $HgCl_2$ before addition to the assay media. The values found varied between 0.2 and 0.5 μmol of [^{32}P]P_i/g of protein (Table I). Except for Table I, the data of E∼P shown under Results were already corrected for unspecific binding of [^{32}P]P_i.

TABLE I: Membrane Phosphorylation by Orthophosphate.[a]

Previous Treatment of SRV	μmol of E∼P/g of Protein
None	2.81 ± 0.23 (14)
6% diethyl ether	2.61 ± 0.27 (10)
$HgCl_2$	0.41 ± 0.04 (13)
5% Cl_3CCOOH	0.42 ± 0.05 (4)
Heated at 100°	0.32 ± 0.02 (4)

[a] The [^{32}P]P_i concentration was 4 mM. Other additions and experimental conditions were as described under Materials and Methods. The incubation time was 30 sec. Previous treatments of SRV: diethyl ether, this was done as described under Materials and Methods; $HgCl_2$, to a suspension of SRV at 15 mg of protein/ml, $HgCl_2$ was added to a final concentration of 20 mM; the suspension was incubated for 10 min in ice before use; Cl_3CCOOH, equal volumes of a suspension of SRV at 10 mg/ml and of Cl_3CCOOH at 10 g% (w/v) were mixed and incubated for 10 min on ice; before use, the denatured protein was washed three times with 10 vol of distilled water; heating at 100°, a suspension of SRV at 10 mg/ml was heated 15 min in boiling water. The values shown represent the average ± the standard error of the number of experiments indicated in parentheses.

Treatment of SRV by Diethyl Ether. Diethyl ether (0.30 ml) was added to a 5-ml suspension of SRV at 10 mg of protein/ml in 10 mM KCl. This mixture was incubated in ice for 20 min before use. This treatment increases the permeability for Ca^{2+} of the SRV membrane and therefore any Ca^{2+} which might have accumulated inside the intact vesicles flows out through these leaky membranes. As a control, in all preparations used, Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity were measured before and after the diethyl ether treatment. In agreement with the data of Inesi *et al.* (1967) and Fiehn and Hasselbach (1969), the SRV treated with diethyl ether no longer accumulated Ca^{2+} although the ATPase activity remained unimpaired. Ca^{2+} uptake and ATPase activity were assayed as previously described (de Meis, 1969, 1971).

Chemicals. [^{32}P]P_i was obtained from the Brazilian Institute of Atomic Energy. All other reagents were of analytical grade.

Results

Phosphorylation of SRV by P_i in the Absence of a Ca^{2+} Concentration Gradient. In order to test the ability of SRV to be phosphorylated in the absence of Ca^{2+}, SRV were incubated in media containing 1 mM EGTA and different [^{32}P]P_i concentrations at pH 6.0 (Figure 1). In the absence of Mg^{2+} only 0.2–0.4 μmol of P_i was incorporated per gram of SRV protein. Upon the addition of Mg^{2+}, the amount of E∼P increased in less than 5 sec to different levels depending on the P_i concentration of the assay media. We were unable to measure the initial rate of E∼P formation in these experiments.

This membrane phosphorylation involves an enzymatic system, since no E∼P formation was observed when the SRV proteins were previously damaged by different procedures (Table I). The data in Table I also show that the membrane phosphorylation by [^{32}P]P_i was not impaired when SRV were made leaky by a previous treatment with 6% diethyl ether.

Characterization of the Phosphorylating Reaction. The amount of E–P formed was measured as a function of the incubation time in media containing 0.4 and 4.0 mM [^{32}P]P_i.

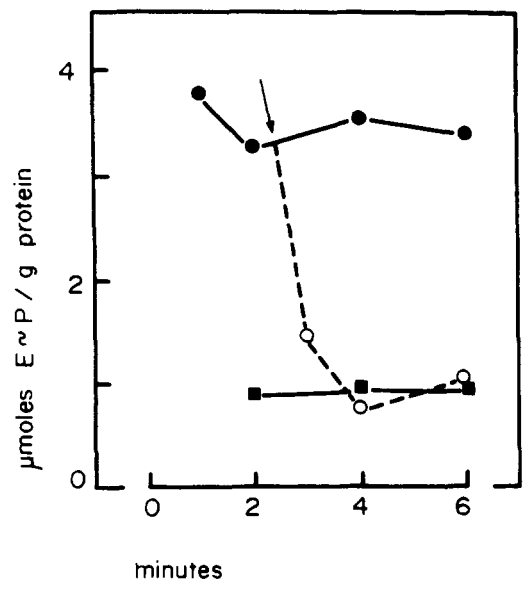

FIGURE 2: Dependence of E~P formation on the P_i concentration. The [^{32}P]P_i concentrations were 4 (●) and 0.4 mM (■). At 2 min and 30 sec incubation (arrow) a sample of the medium containing 4 mM [^{32}P]P_i was diluted to a [^{32}P]P_i concentration of 0.4 mM with a solution containing 10 mM Tris-maleate buffer (pH 6.0), 10 mM $MgCl_2$, and 1 mM EGTA (○- - -○). Other additions and experimental conditions were as described under Materials and Methods. Essentially the same results were obtained in three different SRV preparations tested.

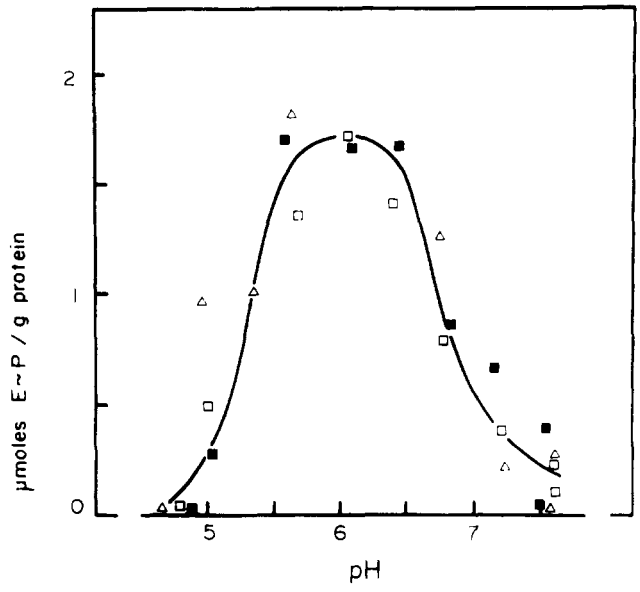

FIGURE 3: pH dependence of E~P formation. The assay medium composition was 1 mM EGTA–10 mM $MgCl_2$–10 mM acetate buffer (pH 4.0–5.0) or Tris-maleate buffer (pH 5.5–8.0) and 4 mM [^{32}P]P_i adjusted to different pH values as shown in the figure. The incubation time was 30 sec. Other additions and experimental conditions were as described under Materials and Methods. Each symbol represents a different SRV preparation tested.

As can be seen in Figure 2, 1.0 and 3.5 μmol of E~P/g of protein were formed, respectively. When an aliquot of the media containing 4.0 mM [^{32}P]P_i was diluted tenfold, the yield of E~P decreased to the range of 1.0 μmol/g of protein. This experiment and the data of Figure 1 suggest that the amount of E~P measured results from a steady-state equilibrium and that the rate of E~P formation is greater than that of hydrolysis.

The pH profile of E~P formation showed a maximum at about pH 6.0 (Figure 3). The Mg^{2+} requirement for membrane phosphorylation was investigated at two concentrations of P_i (Figure 4). Using 5 mM P_i, the ratio Mg^{2+}:P_i for maximal phosphorylation was 2.

In eight different SRV preparations, E~P formation was measured in media containing 10 mM Tris-maleate buffer (pH 6.0), 10 mM $MgCl_2$, and [^{32}P]P_i with concentrations varying from 0.5 to 5.0 mM. The Lineweaver–Burk plot of those data showed that the P_i concentration required for half-maximal phosphorylation was 1.06 ± SE 0.11.

Inhibition of SRV Phosphorylation. Calcium ions strongly inhibit the membrane phosphorylation by [^{32}P]P_i (Figure 5 and Table II); 50% inhibition was obtained with a Ca^{2+} concentration of 10 μM. Figure 6 shows that this inhibition is reversible. If the ionic Ca concentration of the assay media

TABLE II: Inhibition of E~P Formation by Ca^{2+}, Na^+, and K^+.[a]

Additions to Assay Medium	μmol of E~P/g of Protein	% Inhibition
None	2.32 ± 0.36 (18)	
$CaCl_2$, 25 μM	0.20 ± 0.04 (11)	91.4
$CaCl_2$, 100 μM	0.24 ± 0.05 (18)	89.7
KCl, 200 mM	0.85 ± 0.14 (8)	63.4
NaCl, 200 mM	0.63 ± 0.11 (8)	72.8
LiCl, 200 mM	1.66 ± 0.27 (8)	28.4
Sucrose, 400 mM	2.08 ± 0.20 (6)	10.4

[a] The [^{32}P]P_i concentration was 4 mM. Other additions and experimental conditions were as described under Materials and Methods. The incubation time was 20 sec. When $CaCl_2$ was used, EGTA was omitted from the assay medium. The values shown represent the average ± the standard error of the number of experiments indicated in parentheses.

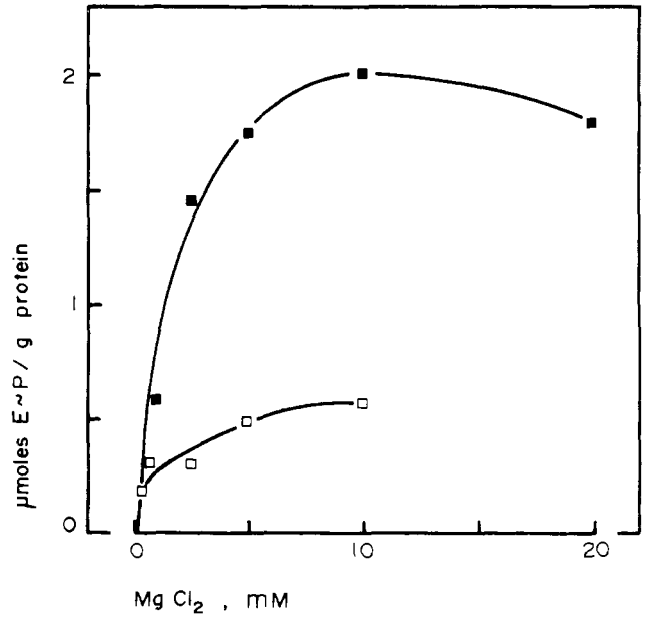

FIGURE 4: Mg^{2+} dependence of E~P formation. The assay medium composition was 10 mM Tris-maleate buffer (pH 6.0), 1 mM EGTA, and 5 (■) or 0.5 mM (□) [^{32}P]P_i. The incubation time was 20 sec. Other additions and experimental conditions were as described under Materials and Methods. Essentially the same results were obtained in five different SRV preparations tested.

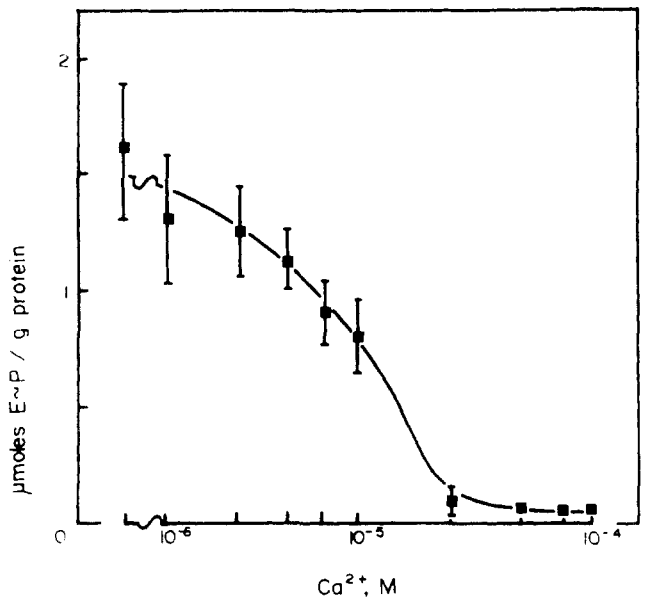

FIGURE 5: Inhibition of E~P formation by Ca^{2+}. The [^{32}P]P_i concentration was 4 mM. For zero Ca^{2+}, no $CaCl_2$ was added and the EGTA concentration was 1.0 mM. For the calculated free Ca^{2+} concentrations of 1.5, 3.0, 5.0, 7.0, and 10.6 μM, 0.2 mM $CaCl_2$ and 0.70, 0.46, 0.35, 0.30, and 0.26 mM EGTA, respectively, were added to the assay medium. For the Ca^{2+} concentrations of 25, 50, and 100 μM, no EGTA was used. The free Ca^{2+} concentration was calculated using the value of 4×10^{-6} for the dissociation constant of the complex Ca-EGTA (de Meis and Hasselbach, 1971). Other additions and experimental conditions were as described under Materials and Methods. The values represent the average ± the standard error (SE) of four experiments.

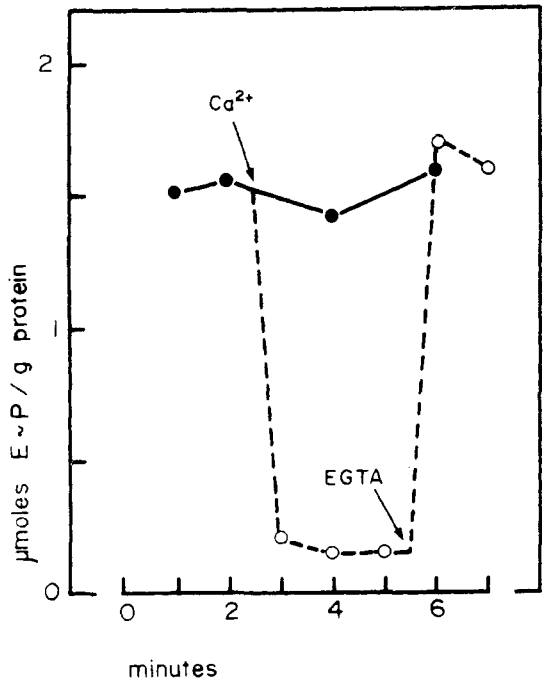

FIGURE 6: The reversible effect of Ca^{2+}. The assay medium composition was 10 mM Tris-maleate buffer (pH 6.0), 0.1 mM EGTA, 10 mM $MgCl_2$, and 4 mM [^{32}P]P_i. To a separate aliquot of this medium, $CaCl_2$ to a final concentration of 0.3 mM and EGTA to a final concentration of 2 mM were added as shown by arrows. Other additions and experimental conditions were as described under Materials and Methods. Essentially the same results were obtained in four different SRV preparations tested.

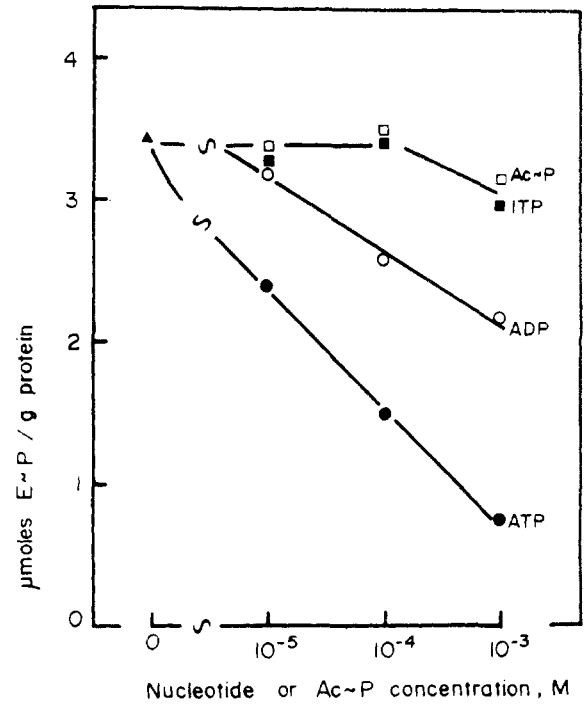

FIGURE 7: Inhibition of E~P formation by nucleotides and acetyl phosphate. The P_i concentration was 4 mM and the incubation time was 15 sec. Other additions and experimental conditions were as described under Materials and Methods: (▲) control assay medium; (●) control assay medium plus ATP; (○) control assay medium plus ADP; (■) control assay medium plus ITP; (□) control assay medium plus acetyl phosphate.

was decreased to less than 1 μM by addition of EGTA, the SRV membrane was quickly rephosphorylated. Na and K ions also inhibit the E~P formation (Table II), 50% inhibition being obtained with the use of 100 mM NaCl. Little inhibition was observed when 200 mM LiCl or 400 mM sucrose were added to the assay medium, indicating that the inhibition observed was due specifically to the cation used, not being related to the ionic strength or to the osmotic balance of the system.

Both ATP and ADP inhibit the membrane phosphorylation by P_i, ATP being more effective than ADP (Figure 7). The data presented in Figure 8 suggest that this inhibition is competitive. ITP, GTP, and acetyl phosphate had little effect on this reaction; AMP and IMP at concentrations up to 4 mM were ineffective as inhibitors.

Characterization of the Phosphorylated Site. The pH stability of the phosphorylated membrane was determined after denaturation by perchloric acid. The E~P was stable in the range of pH 2.0–6.0 and unstable at higher pH.

In four experiments, denaturated E~P was incubated for 15 min at room temperature in 40 mM Tris-maleate buffer (pH 6.4) with and without 0.4 M hydroxylamine (Inesi *et al.*, 1970). After centrifugation, the amounts of E~^{32}P recovered were, respectively, 0.36 ± SE 0.04 and 2.07 ± SE 0.07 μmol/g of protein. These results suggest that the E~P is an acyl phosphate type compound.

Discussion

Several reports have shown that the membrane phosphorylation by a nucleoside triphosphate or acetyl phosphate is only observed in the presence of Ca^{2+} (Makinose, 1969; Friedman and Makinose, 1970; Inesi *et al.*, 1970; Pucell and Mar-

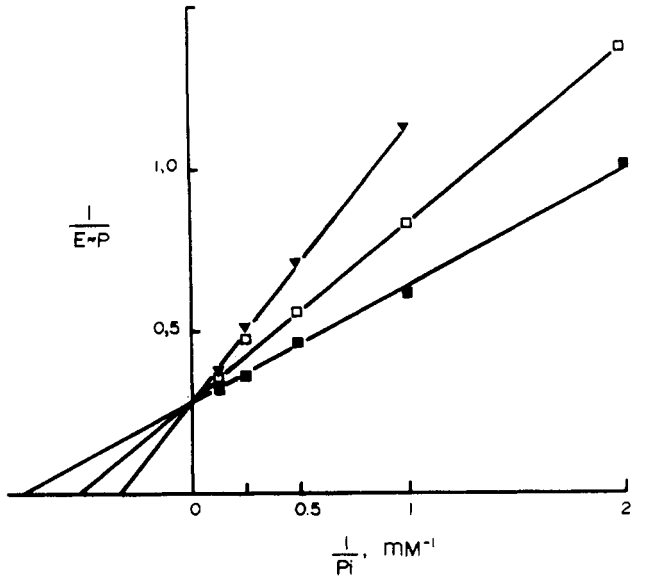

FIGURE 8: Competitive inhibition of E~P formation by ATP and ADP. The assay medium composition was as described under Materials and Methods except that for 7 mM [^{32}P]P_i, the $MgCl_2$ concentration was raised to 20 mM. The incubation time was 10 sec: (■) control assay medium; (□) control assay medium plus 10 μM ATP; (▼) control assay medium plus 100 μM ADP. Essentially the same results were obtained in four different SRV preparations tested.

tonosi, 1971; Panet *et al.*, 1971; de Meis, 1972; de Meis and de Mello, 1973). In this paper it is shown that the membrane phosphorylation by orthophosphate is inhibited by Ca^{2+}. This might suggest that both the nucleosides triphosphate and P_i are substrates of a common system which undergoes a conformational change depending on the binding of Ca^{2+} to the SRV membrane, *i.e.*, the binding of Ca^{2+} would trigger the choice of substrate for the phosphorylating reaction. The following data support this hypothesis: (1) the yield of E~P is in the range of 1.5–3.0 μmol/g of protein when either a nucleoside triphosphate or orthophosphate is used as substrate; (2) the E~P derived from both substrates is an acyl phosphate type compound (Makinose, 1969; Inesi *et al.*, 1970; Yamamoto *et al.*, 1971); (3) ATP competes with P_i for the phosphorylating reaction (Figure 8). In a previous paper (de Meis and de Mello, 1973) it was shown that the K_m for E~P formation of ATP is about 1000 times lower than that of ITP and acetyl phosphate. Accordingly, we found that ITP and acetyl phosphate were very poor inhibitors when compared to ATP (Figure 7). (4) Using a saturating concentration of nucleoside triphosphate, the Ca^{2+} concentration required for half-maximal phosphorylation is in the range of 2–5 μM (de Meis and de Mello, 1973). With the use of P_i, the Ca^{2+} concentration required for half-maximal inhibition of E~P formation is 10 μM. These two values are quite close. (5) Finally, several reports have presented evidence that the Ca^{2+} transport system has characteristics similar to those described for allosteric enzymes (Yamamoto and Tonomura, 1967; Inesi *et al.*, 1967, 1970; de Meis and de Mello, 1973).

Makinose (1972) has shown that during Ca^{2+} efflux, the SRV membrane is phosphorylated by orthophosphate provided that a steep Ca^{2+} concentration is formed across the membrane. In this paper we have shown that this reaction is also observed in the absence of a Ca^{2+} concentration gradient. Using the experimental conditions described by Makinose (1972), we were able to reproduce his data. At present we cannot precisely determine whether or not the E~P described by Makinose and the E~P described in this paper are formed in the same membrane site. Experiments on this problem are now in progress.

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