# Phosphorylation of the Sarcoplasmic Reticulum Membrane by Orthophosphate through Two Different Reactions†

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ABSTRACT: A protein of the sarcoplasmic reticulum membrane of skeletal muscle is phosphorylated by orthophosphate through two different reactions. One of these reactions is strongly inhibited by $Ca^{2+}$ and is not modified when acetyl phosphate, ITP, or GTP is added to the assay medium. The other reaction is observed in the presence of $Ca^{2+}$ and requires ITP, GTP, or acetyl phosphate as cofactors. The two reactions have a different pH profile and are inhibited by ATP and ADP to different extents. In presence of $Ca^{2+}$, both ITP and orthophosphate phosphorylate a common site of the membrane. A reaction sequence is proposed on the basis of the data presented.

Sarcoplasmic reticulum vesicles (SRV)[1] isolated from skeletal muscle retain a highly efficient ATP-dependent $Ca^{2+}$ transport system (Hasselbach and Makinose, 1961; Hasselbach, 1964). In the process of ATP hydrolysis, the $\gamma$-phosphate of ATP is covalently bound to a membrane protein (E). This phosphoprotein (E-P) represents an intermediary product in the sequence of reactions leading to $Ca^{2+}$ transport and $P_i$ liberation (Makinose, 1966, 1969; Yamamoto and Tonomura, 1967). Different NTP, AcP or *p*-nitrophenyl phosphate can substitute for ATP as substrate for the active $Ca^{2+}$ transport (de Meis, 1969; Friedman and Makinose, 1970; Inesi, 1971; Pucell and Martonosi, 1971). The $K_m$ for E-P formation by ATP, ITP, GTP, and AcP are respectively $2.4 \times 10^{-}$,[8] $3.1 \times 10^{-6}$, $4.5 \times 10^{-5}$, and $3.8 \times 10^{-4}$ M (de Meis and de Mello, 1973).

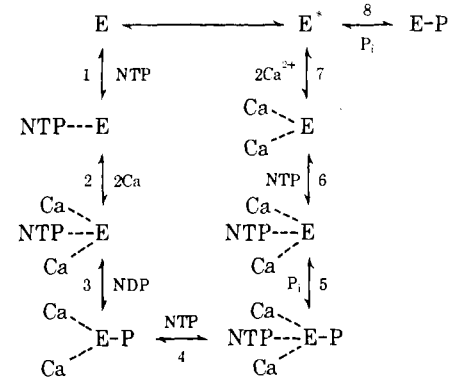
Recently it has been shown that under specific conditions, the $Ca^{2+}$ pump of the SRV can be reversed. When SRV previously loaded with calcium oxalate or calcium phosphate is incubated in a medium containing ADP, $Mg^{2+}$, and [$^{32}$P]$P_i$, it is observed that [$^{32}$P]$P_i$ interacts with the membrane, forming E-P (Makinose, 1972), $Ca^{2+}$ is released at a very high rate (Barlogie *et al.*, 1971) and [$\gamma$-$^{32}$P]ATP is formed (Makinose, 1971, 1973; Makinose and Hasselbach, 1971; Yamada *et al.*, 1972). In these experiments evidence has been presented 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.

In a previous paper (Masuda and de Meis, 1973) it has been shown that [$^{32}$P]$P_i$ can react with the SRV forming a high-energy E-P in the absence of a $Ca^{2+}$ concentration gradient. This reaction is strongly inhibited by $Ca^{2+}$, therefore differing from the membrane phosphorylation promoted by NTP which is activated by $Ca^{2+}$. Similar findings were reported by Kanazawa and Boyer (1973). These authors also observed that SRV catalyze a rapid $P_i \rightleftharpoons HOH$ exchange which is inhibited by $Ca^{2+}$.

In this paper it is shown that $Ca^{2+}$ inhibition of the membrane phosphorylation by [$^{32}$P]$P_i$ is abolished when a substrate of low affinity such as ITP or AcP is included in the system. While this paper was in preparation, Makinose (1973) reported that the SRV membrane can be simultaneously phosphorylated by [$\gamma$-$^{32}$P]ITP and [$^{32}$P]$P_i$.

The results reported were temptatively interpreted according to Scheme I.

SCHEME I




## Materials and Methods

*Sarcoplasmic Reticulum Vesicles.* These were prepared from rabbit skeletal muscle as previously described (de Meis and Hasselbach, 1971).

*[$\gamma$-$^{32}$P]ITP.* This was prepared as previously described (de Meis and de Mello, 1973).

*$^{32}P_i$.* This was obtained from the Brazilian Institute of Atomic Energy and purified by means of a column of Dowex AG1-X8 resin (mesh 200–400) previously treated with 1 N NaOH. After the addition of the [$^{32}$P]$P_i$ solution, the column was washed with deionized water and the [$^{32}$P]$P_i$ was eluted with 0.04 N HCl. The [$^{32}$P]$P_i$ was stored in a 0.04 N HCl solution until used.

*Standard Assay.* The incubation medium composition is described in the figure legends. The $P_i$ solutions were adjusted to the same pH as the buffer used. For measuring E-P formation the reaction was started by the addition of SRV. When

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[1] Abbreviations used are: SRV, sarcoplasmic reticulum vesicles; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; AcP, acetyl phosphate; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N,N'*-tetraacetic acid.

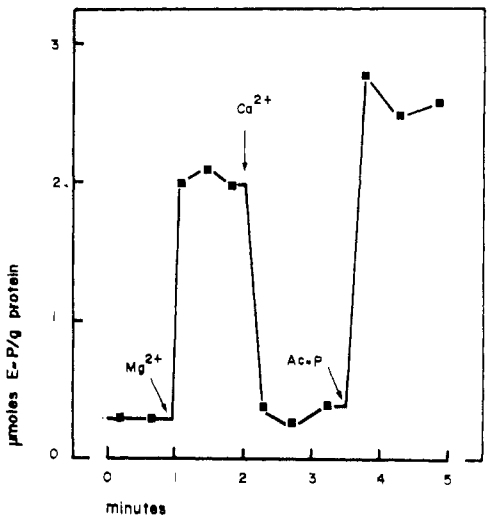


FIGURE 1: Effect of $Ca^{2+}$ and acetyl phosphate on E-P formation. The assay medium composition was 10 mM Tris–maleate buffer (pH 6.5), 4 mM [$^{32}$P]$P_i$, 0.05 mM EGTA, and 0.6 mg of SRV protein per ml. The reaction was started by the addition of SRV. After different incubation intervals at 37°, aliquots of 2.5 ml of this medium were removed for E-P determination. Arrows point to the addition of $MgCl_2$, final concentration in the assay medium of 10 mM; $CaCl_2$, 0.25 mM; AcP, 2 mM. Essentially the same results were observed in three different SRV preparations tested.

the volume of the assay medium was 2.5 ml, the reaction was stopped by injecting it into 20 ml of an ice-cold solution of 125 mM perchloric acid containing 2 mM orthophosphate. When the volume of the assay medium was 20 ml, the reaction was stopped by adding 1.3 ml of an ice-cold solution of trichloroacetic acid at 100% (w/v) containing 20 mM orthophosphate, while stirring vigorously at 0°. The resulting 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 planchette 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 were denaturated by 20 mM $HgCl_2$ before addition to the assay media. The values found varied between 0.2 and 0.4 μmol of [$^{32}$P]$P_i$ per g of protein. Except for Figure 1, the data of E-P shown in Results were already corrected for unspecific binding of [$^{32}$P]$P_i$.

*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).

## Results

*Membrane Phosphorylation by [$^{32}$P]$P_i$ in the Presence of $Ca^{2+}$.* When SRV was incubated in media containing [$^{32}$P]$P_i$, 0.2–0.4 μmol of [$^{32}$P]$P_i$ was incorporated per g of SRV protein (Figure 1). Upon the addition of $Mg^{2+}$, the amount of E–P increases in less than 5 sec to the range of 1.5–3.0 μmol/g of protein. If $Ca^{2+}$ was added to the assay medium, the E–P formation was completely inhibited. The membrane phosphorylation by $P_i$ and its inhibition by $Ca^{2+}$ has been further documented in a previous paper (Masuda and de Meis, 1973), and would correspond to steps 8 and 7 in the reaction sequence proposed in the introduction. If after the inhibition by $Ca^{2+}$, Ac–P, ITP, or GTP was added to the assay media, the membrane was again rephosphorylated by [$^{32}$P]$P_i$ (Figure 1 and Table I). ATP in the concentration range of 10 μM to 1 mM failed to abolish the $Ca^{2+}$ inhibition. In the presence of Ac–P, the membrane phosphorylation by [$^{32}$P]$P_i$ was not impaired by a large range of $Ca^{2+}$ concentrations (Figure 2). In the reaction sequence, this phosphorylation would correspond to steps 6 and 5. For these reactions $Mg^{2+}$ is required as activating ion and the steady-state level of E–P varies with the [$^{32}$P]$P_i$ concentration of the assay medium (Figure 3).

Figure 4 shows that different pH profiles of E–P formation were observed whether the membrane was phosphorylated by [$^{32}$P]$P_i$ in the absence of $Ca^{2+}$ (step 8), by [$^{32}$P]$P_i$ in presence of $Ca^{2+}$ and ITP (step 5) or by [γ-$^{32}$P]ITP (step 3).

*Simultaneous Phosphorylation of the Membrane by ITP and $P_i$.* In presence of $Ca^{2+}$, ITP phosphorylates the SRV membrane (de Meis and de Mello, 1973). The following set of experiments was designed in order to ascertain whether $P_i$ and ITP phosphorylate a common protein of the membrane.

Table II shows that the yield of E–$^{32}$P was essentially the same when SRV was incubated with 0.2 mM $CaCl_2$ and either 0.2 mM [γ-$^{32}$P]ITP alone or with 0.2 mM [γ-$^{32}$P]ITP plus 4 mM [$^{32}$P]$P_i$. In media containing both substrates where only one was radioactive, the yield of E–$^{32}$P in each individual experiment decreased but, the sum of the two values found was equal to the E–$^{32}$P yield obtained when both substrates were radioactive. This finding was further developed as shown in Figure 5 using 0.2 mM $Ca^{2+}$ and different ITP concentrations.

TABLE I: Membrane Phosphorylation by [$^{32}$P]$P_i$ in Presence of $Ca^{2+}$.[a]

| Additions to Assay Medium | μmol of E–P/g of Protein |
|---|---|
| 0.1 mM EGTA | 2.13 ± 0.18 (22) |
| 0.2 mM $CaCl_2$ | 0.30 ± 0.05 (20) |
| 0.2 mM $CaCl_2$ plus 2.0 mM AcP | 2.25 ± 0.15 (24) |
| 0.2 mM $CaCl_2$ plus 0.1 mM ITP | 2.52 ± 0.16 (21) |
| 0.2 mM $CaCl_2$ plus 1.0 mM ITP | 1.89 ± 0.19 (13) |
| 0.2 mM $CaCl_2$ plus 0.1 mM GTP | 2.79 ± 0.09 (2) |
| 0.2 mM $CaCl_2$ plus 1.0 mM GTP | 1.81 ± 0.30 (4) |
| 0.2 mM $CaCl_2$ plus 0.01 mM ATP | 0.13 ± 0.04 (4) |
| 0.2 mM $CaCl_2$ plus 1.0 mM ATP | 0.04 ± 0.02 (4) |

[a] The assay medium composition was 10 mM Tris–maleate buffer (pH 6.5), 10 mM $MgCl_2$, 2 mM [$^{32}$P]$P_i$, and 0.5 mg of SRV protein per ml. Other additions were as shown in the table. The reaction was performed at 37° and the incubation time was 20 sec. The values represent the average ±SE of the number of experiments indicated in parentheses.

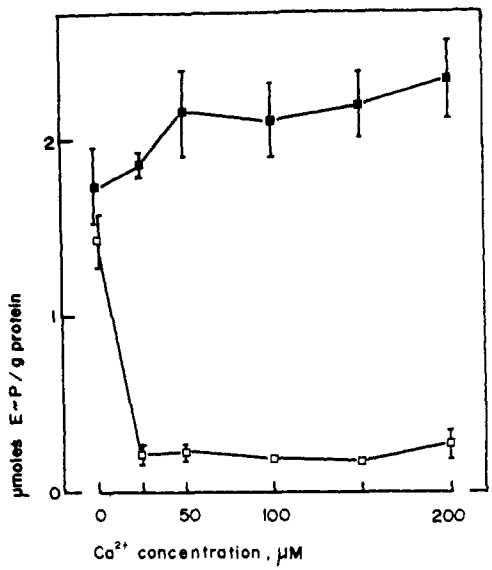


FIGURE 2: Inhibition of E–P formation by $Ca^{2+}$. The assay medium composition was 10 mM Tris–maleate buffer (pH 6.5), 4 mM [$^{32}$P]$P_i$, 10 mM $MgCl_2$, and 0.6 mg of SRV protein per ml. For the point referring to zero $Ca^{2+}$, 0.1 mM EGTA was included in the medium. For the different $Ca^{2+}$ concentrations, EGTA was omitted and $CaCl_2$ was added in the concentrations shown in the figure. The final volume was 2.5 ml. The reaction was started by the addition of SRV and stopped as described in Methods after 15-sec incubation at 37°: (□) without AcP; (■) with 2 mM AcP. The values represent the average ±SE of eight experiments.

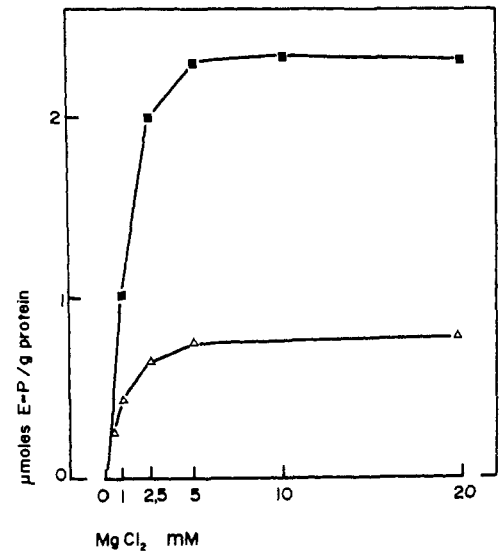


FIGURE 3: $Mg^{2+}$ dependence of E–P formation. The assay medium composition was 10 mM Tris–maleate buffer (pH 6.8), 0.2 mM $CaCl_2$, 0.1 mM ITP, 0.6 mg of SRV protein per ml, and different $MgCl_2$ concentrations as shown in the figure. The final volume was 2.5 ml. The reaction was started by the addition of SRV and stopped after 20-sec incubation at 37°: (△) 0.5 mM [$^{32}$P]$P_i$; (■) 4.0 mM [$^{32}$P]$P_i$. Essentially the same results were obtained in four different SRV preparations tested.

In this experiment, the yield of E–$^{32}$P obtained with [$\gamma$-$^{32}$P]ITP in the absence of $P_i$ was measured and was assumed to correspond to 100% of the phosphorylating sites of the membrane. Figure 5 shows that with higher concentration ratios of ITP:$P_i$ in the assay medium, a higher percentage of the membrane sites was phosphorylated by [$\gamma$-$^{32}$P]ITP and a lower percentage was phosphorylated by [$^{32}$P]$P_i$.

The double-reciprocal plot shown in Figure 6 shows that excess of ITP inhibits competitively the membrane phosphorylation by [$^{32}$P]$P_i$. Using 0.1 mM ITP and 0.2 mM $Ca^{2+}$, the [$^{32}$P]$P_i$ concentration required for half-maximal membrane phosphorylation was 1.4 mM ± SE 0.15 (six experiments).

*Inhibition of E–P Formation by ATP and ADP.* Figure 7 shows that ATP and ADP sharply inhibit the membrane phosphorylation by [$^{32}$P]$P_i$ in presence of $Ca^{2+}$ and ITP. AMP, GDP, IDP, IMP, and GMP, up to 1 mM concentration, had little or no effect in this reaction.

TABLE II: Competition of ITP and $P_i$ for a Common Phosphorylating Site on the Membrane.[a]

| Additions to Assay Medium | μmol of E–$^{32}$P/g of Protein |
|---|---|
| 0.2 mM [$\gamma$-$^{32}$P]ITP | 3.93 ± 0.11 |
| 0.2 mM [$\gamma$-$^{32}$P]ITP plus 4 mM [$^{32}$P]$P_i$ | 3.58 ± 0.18 |
| 0.2 mM [$\gamma$-$^{32}$P]ITP plus 4 mM $P_i$ | 1.58 ± 0.09 |
| 0.2 mM ITP plus 4 mM [$^{32}$P]$P_i$ | 2.01 ± 0.20 |

[a] The assay medium composition was 10 mM Tris–maleate buffer (pH 6.7), 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 0.2 mg of SRV protein per ml. The reaction was performed at 37° and the incubation time was 20 sec. The [$\gamma$-$^{32}$P]ITP and [$^{32}$P]$P_i$ solutions were adjusted to the same specific activity. The values represent the average ± SE of eight experiments.

*$Ca^{2+}$ Concentration Gradient.* The experiments of Figure 8 and Table III were designed in order to ascertain whether the membrane phosphorylation by [$^{32}$P]$P_i$ in presence of ITP and $Ca^{2+}$, was related to a $Ca^{2+}$ concentration gradient formed across the SRV membrane. Figure 8 shows an experiment where the $Ca^{2+}$ uptake by SRV and membrane phosphorylation by [$^{32}$P]$P_i$ were simultaneously measured. During the initial 10-min incubation, different $Ca^{2+}$ concentration gradients were formed across the SRV membrane. However,

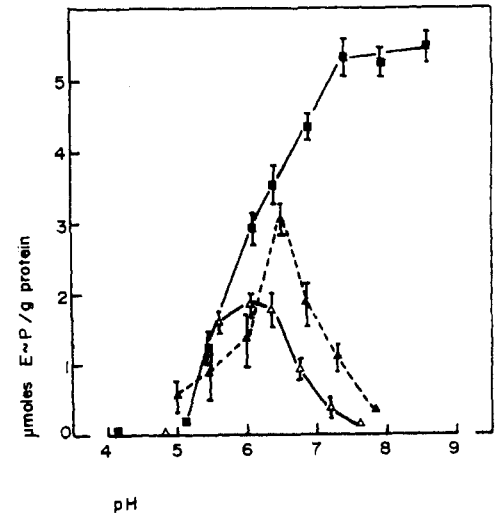


FIGURE 4: pH dependence of membrane phosphorylation. The assay medium compositon was 10 mM acetate buffer (pH 4.0–5.0), Tris–maleate buffer (pH 5.5–8.0) or Tris-HCl (pH 8.5–9.0), 10 mM $MgCl_2$ and (■) 0.2 mM $CaCl_2$, 0.1 mM [$\gamma$-$^{32}$P] ITP, and 0.2 mg of SRV protein per ml; (▲) 0.2 mM $CaCl_2$, 0.1 mM nonradioactive ITP, 4 mM [$^{32}$P]$P_i$, and 0.6 mg of SRV protein per ml. (△) 1 mM EGTA, 4 mM [$^{32}$P]$P_i$ and 0.5 mg of SRV protein per ml. The pH of the [$^{32}$P]$P_i$ solutions was adjusted to different values as shown in the figure. The reactions were performed at 37° and the incubation time was 20 sec. The values represent the average ±SE of four experiments.

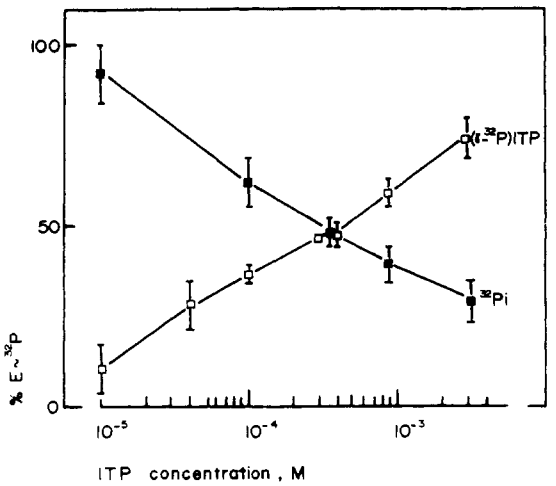


FIGURE 5: Membrane phosphorylation by [$\gamma$-$^{32}$P]ITP and [$^{32}$P]$P_i$. The assay medium composition was 10 mM Tris–maleate buffer (pH 6.7), 4 mM $P_i$, 8 mM $MgCl_2$, 0.2 mM $CaCl_2$, and different ITP concentrations as shown in the figure. For ITP concentrations of 10 and 40 $\mu$M, the SRV protein concentration was 0.07 mg/ml, for the ITP concentrations in the range of 0.1–0.4 mM, the SRV protein was 0.2 mg/ml and for ITP concentrations of 1 and 4 mM, it was 0.5 mg/ml. The incubation time was 15 sec and the reaction was performed at 37°: (□) [$\gamma$-$^{32}$P]ITP and nonradioactive $P_i$; (■) [$^{32}$P]$P_i$ and nonradioactive ITP. For each ITP concentration, control experiments were performed using the same assay medium as □ but omitting the $P_i$. In these experiments the yield of E–$^{32}$P varied between 3 and 4 $\mu$mol per g of protein independently of the [$\gamma$-$^{32}$P]ITP concentration in the assay medium (de Meis and de Mello, 1973). The per cent of the membrane sites phosphorylated by [$\gamma$-$^{32}$P]ITP (□) or by [$^{32}$P]$P_i$ (■) was calculated assuming that the yield of E–$^{32}$P obtained in the control experiments corresponded to 100% of the membrane phosphorylating sites. The values shown in the figure represent the average ±SE of four experiments.

the E–P formation did not vary. It is to be noted that we were not able to measure the E–P formation in the first seconds of incubation where no $Ca^{2+}$ was accumulated by the vesicles. Therefore in this experiment we did not have the optimal control which would be the E–P formation in the absence of any $Ca^{2+}$ concentration gradient. The data of Table III show that when the SRV were made leaky by means of diethyl ether, the membrane was still phosphorylated by [$^{32}$P]$P_i$. However, the yield of E–P was significantly lower than that obtained with control preparations not treated by ether.

TABLE III: Effect of Diethyl Ether.[a]

| SRV Treatment | $Ca^{2+}$ Uptake ($\mu$mol of $Ca^{2+}$/mg of Protein) | Membrane Phosphorylation by [$^{32}$P]$P_i$ ($\mu$mol of E–$^{32}$P/g of Protein) |
|---|---|---|
| None | 1.57 ± 0.05 | 1.80 ± 0.07 |
| 6% diethyl ether | 0 | 0.77 ± 0.07 |

[a] The assay medium composition and experimental conditions were as described in Figure 8. For $Ca^{2+}$ uptake the incubation time was 10 min. For E–P formation, essentially the same results were obtained when the incubation time was varied from 1 to 10 min. Treatment of the SRV with diethyl ether was performed as described under Methods. The values represent the average ±SE of eight experiments.

*Characterization of the Phosphorylated Site.* The effect of hydroxylamine and the pH stability of the phosphorylated membrane were determined after denaturation by trichloroacetic acid. The data of Figure 9 and Table IV suggest that the E–P formed by [$^{32}$P]$P_i$ in the presence or absence of $Ca^{2+}$ and ITP were both acyl phosphate type compounds (Makinose, 1969; Inesi *et al.*, 1970; Dahms *et al.*, 1973).

## Discussion

*Reaction Sequence.* The data presented show that the SRV membrane can be phosphorylated by [$^{32}$P]$P_i$ through two different reactions, which were tentatively represented as reactions 8 and 5. They can be distinguished by the following observations: (a) reaction 8 is strongly inhibited by $Ca^{2+}$, 50% inhibition being obtained with a $Ca^{2+}$ concentration of 10 $\mu$M (Masuda and de Meis, 1973; Kanazawa and Boyer, 1973) while reaction 5 is not impaired by $Ca^{2+}$ (Figure 2); (b) reaction 8 is observed in the absence of ITP or Ac–P, and the addition of these compounds to the assay medium in the concentration range of 10 $\mu$M to 1 mM does not modify the level of E–P formation (Masuda and de Meis, 1973). For reaction 5, ITP or Ac–P is required (Figure 1). Excess of ITP competes with $P_i$ for the phosphorylating site of the membrane (Figures 5 and 6); (c) the two reactions show a different pH profile (Figure 4); (d) both reactions are inhibited by ADP,

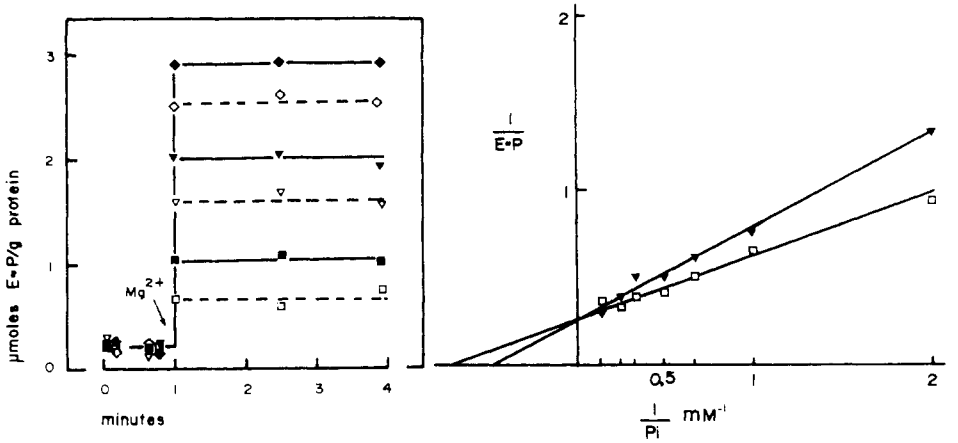


FIGURE 6: Inhibition of E–P formation by excess of ITP. Left: the assay medium composition was 10 mM Tris–maleate buffer (pH 6.5), 0.2 mM $CaCl_2$, 0.6 mg of SRV protein per ml, and 0.1 mM ITP (closed symbols) or 1.0 mM ITP (open symbols). Arrow points to the addition of $MgCl_2$, final concentration in the assay medium of 10 mM. The reaction was performed at 37°: (□,■) 0.5 mM [$^{32}$P]$P_i$; (▽,▼) 1.5 mM [$^{32}$P]$P_i$; (◇,◆) 4.0 mM [$^{32}$P]$P_i$. Right: double-reciprocal plot of the steady-state values of E–P as obtained in the left-hand figure: (□) 0.1 mM ITP; (▼) 1.0 mM ITP. Essentially the same results were observed in six different SRV preparations tested.

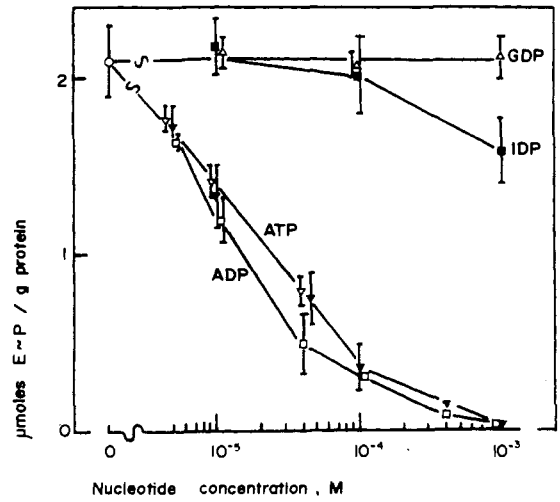


FIGURE 7: Inhibition of E–P formation by ATP and ADP. The assay medium composition was 10 mM Tris–maleate (pH 6.5), 10 mM $MgCl_2$, 3.5 mM [$^{32}$P]$P_i$, 0.2 mM $CaCl_2$, 0.1 mM ITP, and 0.6 mg of SRV protein per ml. The reaction was performed at 37° and the incubation time was 15 sec: (△) GDP; (■) IDP; (▽) ATP; (▼) ATP plus 0.5 mM phosphoenolpyruvate and 30 μg/ml of pyruvic phosphoferase (EC 3.7.1.40); (□) ADP. The values shown in the figure represent the average ±SE of four experiments.

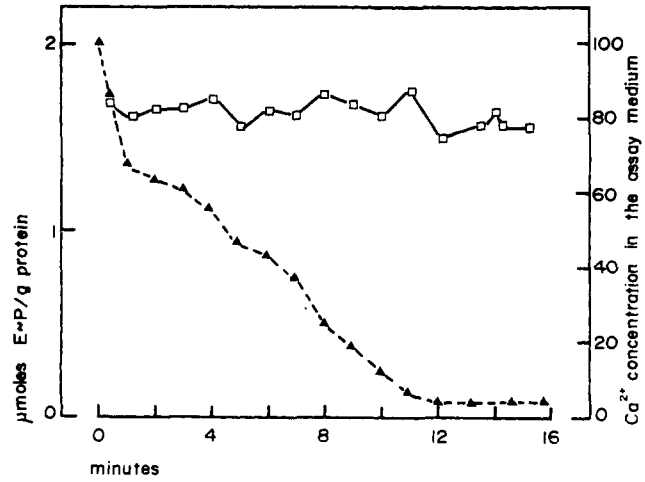


FIGURE 8: E–P formation with varying $Ca^{2+}$ concentration gradient. The assay medium composition was Tris–maleate buffer (pH 6.8), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 4 mM potassium oxalate, 4 mM [$^{32}$P]$P_i$, 2 mM AcP, and 0.5 mg of SRV protein per ml. For measuring $Ca^{2+}$ uptake, $^{45}$Ca and nonradioactive $P_i$ were used. For E–P determination, [$^{32}$P]$P_i$ and nonradioactive $Ca^{2+}$ were used. The reaction was performed at 28°: (▲) $Ca^{2+}$ uptake by SRV; (□) membrane phosphorylation by [$^{32}$P]$P_i$. The right-hand abscissa represents the percentage of $Ca^{2+}$ remaining in the solution.

but using 4 mM [$^{32}$P]$P_i$, the ADP concentration required to obtain 50% inhibition was 2–4 mM for reaction 8 (Masuda and de Meis, 1973) and 0.01–0.02 mM for reaction 5 (Figure 7).

In a previous paper, the membrane phosphorylation by different NTPs was studied (de Meis and de Mello). Evidence was presented that the NTP, besides being a substrate for E–P formation (reaction 3) also regulates the $Ca^{2+}$ affinity of the enzyme (reactions 1 and 2) and activates the rate of E–P hydrolysis (reaction 4). These reactions were further discussed in the paper cited above. At present we have no evidence whether the nucleotides involved in reactions 1 and 6 bind to a common site on the membrane or, whether they bind in different sites. The same apply for the binding of $Ca^{2+}$ in reactions 2 and 7.

In the reaction sequence it was postulated that the phosphorylating structure of the membrane could be found in two different interconvertible forms, E and E*. The form E would be phosphorylated by NTP and the form E* by $P_i$. This hypothesis is supported by the following observations:

TABLE IV: Effect of Hydroxylamine.[a]

| Additions to the Phosphorylating Media | μmol of E–$^{32}$P/g of Protein | |
|---|---|---|
| | Without Hydroxylamine | +0.4 M Hydroxylamine |
| 0.2 mM EGTA | 2.07 ± 0.07 | 0.36 ± 0.04 |
| 0.2 mM $CaCl_2$ and 0.1 mM ITP | 1.72 ± 0.02 | 0.26 ± 0.07 |

[a] SRV membrane phosphorylated by [$^{32}$P]$P_i$ in presence of EGTA at pH 6.0 or at pH 6.5 in presence of 0.2 mM $Ca^{2+}$ and 0.1 mM ITP were prepared as described in Figure 4. After denaturation by trichloroacetic acid, the protein pellets were washed three times with a 125 mM perchloric acid solution containing 2 mM $P_i$ and once with 80 ml of ice-cold water. Aliquots of the denatured proteins were incubated at room temperature in 40 mM citrate buffer (pH 5.5) with and without 0.4 M hydroxylamine during 10 min. The protein concentration in the buffer solution was 0.1 mg/ml. After centrifugation, the amount of radioactive E–P recovered in the pellet was determined as described under Methods. The values represent the average ±SE of four experiments.

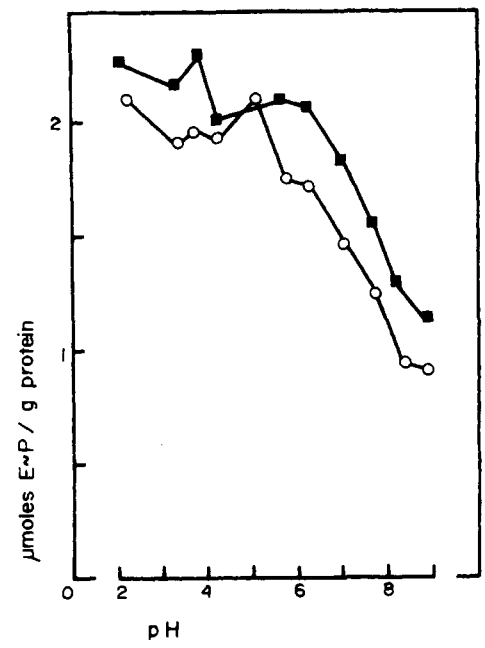


FIGURE 9: Nonenzymatic degradation of E–P at different pH's. SRV membrane phosphorylated by [$^{32}$P]$P_i$ in the absence of $Ca^{2+}$ at pH 6.0 (○) or at pH 6.5 in presence of 0.2 mM $Ca^{2+}$ and 0.1 mM ITP (■) were prepared as described in Figure 4. After denaturation by trichloroacetic acid, the protein pellets were washed three times with a 125 mM perchloric acid solution containing 2 mM nonradioactive $P_i$. In order to remove the excess of perchloric acid, the precipitates were washed twice with 80 ml of ice-cold distilled water. Aliquots of the denaturated proteins were exposed for 30 min at room temperature to different pH's (40 mM citrate, acetate, Tris–maleate, Tris-HCl buffers). The protein concentration in the buffer solution was 0.1 mg/ml. After centrifugation, the amount of radioactive E–P recovered in the pellet was determined as described under Methods. The data shown in the figure represent the average of two experiments.

(a) at pH 8, the membrane is fully phosphorylated by ITP but is not by $P_i$ (Figure 4); (b) in a previous paper (de Meis and de Mello, 1973) it was shown that using different NTP as phosphorylating substrates, the steady-state level of E–P is determined by the $Ca^{2+}$ concentration in the assay medium and not by the substrate concentration. Using $P_i$ as the phosphorylating substrate the steady-state level of E–P is determined by the $P_i$ concentration in the assay medium both when the phosphorylation proceeds through reaction 8 (Masuda and de Meis, 1973) or through reaction 5 (Figures 3 and 6 left).

*Inhibition by ITP and ATP.* In the presence of $Ca^{2+}$, ITP operates both as an activator of the membrane phosphorylation by $P_i$ (Figure 1) and as a phosphorylating substrate (Table II). As a working hypothesis it is postulated that $[^{32}P]P_i$ would only be able to react with the species E* or NTP: $E<^{Ca}_{Ca}$ and not with $E<^{Ca}_{Ca}$. This would account for the activating effect of ITP, GTP, and AcP. The competitive effect of ITP with $[^{32}P]P_i$ observed in Figure 6 probably results from the simultaneous membrane phosphorylation by ITP through reaction 3. With higher ratios of ITP:$P_i$ in the assay medium, a larger number of sites would be phosphorylated by ITP and a lower number of sites would be left available to be phosphorylated by $P_i$.

In a previous paper (de Meis and de Mello, 1973), it was shown that the apparent $K_m$ of ATP for the phosphorylating site is 1200 to 1800 times lower than that of ITP or GTP and about 16,000 times lower than for AcP. These differences of affinity probably account for the data of Table I and Figure 7. Even when the ATP:$P_i$ ratio in the assay medium was very low, most of the membrane sites would be phosphorylated by ATP.

*$Ca^{2+}$ Concentration Gradient.* In a previous paper it was shown that reaction 8 is independent of the formation of a $Ca^{2+}$ concentration gradient through the SRV membrane. For reactions 5 and 6, the data of Table III and Figure 8 do not permit a similar clear conclusion. At present we have no data to clarify this apparent discrepancy. It might be possible that reactions 5 and 6 can occur in the absence of a $Ca^{2+}$ concentration gradient and that the establishment of a gradient would enhance the level of E–P formation (Table III). The data of Figure 8 might imply that the gradient formed in the first seconds of incubation would be already large enough to cause maximum activation of membrane phosphorylation by $P_i$.

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