A New Mechanism by Which an H^+ Concentration Gradient Drives the Synthesis of Adenosine Triphosphate, pH Jump, and Adenosine Triphosphate Synthesis by the Ca^{2+}-Dependent Adenosine Triphosphatase of Sarcoplasmic Reticulum†

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ABSTRACT: The Ca^{2+}-dependent ATPase purified from the sarcoplasmic reticulum of skeletal muscle synthesizes ATP in the absence of a Ca^{2+} concentration gradient. This is attained in a two-step procedure in which the enzyme is initially phosphorylated by P_i in the absence of Ca^{2+}, and then the phosphate is transferred to ADP by the addition of high levels of Ca^{2+}. The degree of enzyme phosphorylation by P_i varies with the Ca^{2+} concentration and pH of the assay medium. At pH 6.0, 50% inhibition is observed in the presence of 10 to 20 μM Ca^{2+}. At pH 5, while the level of phosphoenzyme is reduced, 50% inhibition is only observed in the presence of 1 to 2 mM Ca^{2+}. The transfer of phosphate from the phosphoenzyme to ADP is dependent upon the saturation of a low-affinity Ca^{2+}-binding site. After phosphorylation by P_i, it is possible to transfer the phosphate to ADP at progressively lower Ca^{2+} concentrations as the pH is raised from 6.0 to 8.1. Maximal ATP synthesis is attained at a Ca^{2+} concentration range of 1 to 2 mM at pH 8.1, compared with more than 40 mM at pH 6.0. When the enzyme is phosphorylated at pH 5.0 in the presence of 0.6 mM $CaCl_2$, net synthesis of ATP is observed if at the time of ADP addition the pH is rapidly increased to 8.0. The synthesis of ATP was found to correlate with the dephosphorylation of phosphoenzyme and study of the time course of these reactions revealed identical time constants, the half-time for each being in the range of 30–40 ms. The rapid dephosphorylation of phosphoenzyme was only observed upon the addition of both ADP and high concentrations of Ca^{2+}, dephosphorylation by Ca^{2+} alone being about 100 times slower.

Under appropriate conditions, the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum vesicles can build up a Ca^{2+} concentration gradient across the membrane at the expense of ATP hydrolysis and, once formed, the gradient can be used by the enzyme to synthesize ATP (Hasselbach, 1974; MacLennan and Holland, 1975). The synthesis of ATP was first demonstrated by Barlogie et al. (1971), Makinose (1971, 1972), and Makinose and Hasselbach (1971). When vesicles preloaded with calcium are incubated in a medium containing EGTA,[1] the Ca^{2+}-dependent ATPase is phosphorylated by P_i forming an acylphosphoprotein (E~P) and this is then able to transfer its phosphate to ADP. Coupled with the synthesis of ATP, Ca^{2+} is released by the vesicles at a fast rate. On the grounds of the experiments reported, Makinose (1972) and Yamada et al. (1972) concluded that the osmotic energy derived from the Ca^{2+} concentration gradient across the membrane was used by the system to form an energy-rich E~P.

Work in this laboratory has been aimed at the understanding of the mechanism by which the calcium gradient is used for the synthesis of ATP. Masuda and de Meis (1973) showed that $^{32}P_i$ could react with the transport enzyme forming an acylphosphoprotein in the absence of a calcium concentration gradient, demonstrating that energy derived from the gradient is not required for E~P formation. However, without a gradient the system is not able to catalyze the synthesis of ATP; i.e., E~P is not able to transfer the phosphate to ADP (de Meis, 1976).

The phosphorylation of the enzyme by P_i is inhibited by the binding of calcium at a site of high affinity (apparent K_m, 1 to 3 μM at pH 7.0) located on the outer surface of the membrane (Masuda and de Meis, 1973; Kanazawa, 1975; Knowles and Racker, 1975; de Meis, 1976).

The ATP $\rightleftharpoons$ P_i exchange reaction was first reported by Makinose (1971) who showed that in conditions where a Ca^{2+} concentration gradient is formed the vesicles catalyze a steady exchange between P_i and the γ-phosphate of ATP. Subsequent studies revealed that an ATP $\rightleftharpoons$ P_i exchange is catalyzed by vesicles in the absence of a calcium gradient provided that the low affinity calcium-binding site (apparent K_m, 1–3 mM at pH 7.0) located in the inner surface of the membrane is saturated. This experimental approach provided evidence that after phosphorylation by P_i E~P is only able to transfer its phosphate to ADP when Ca^{2+} binds to this site of low affinity (de Meis and Carvalho, 1974; de Meis and Sorenson, 1975; Carvalho et al., 1976). In conditions where the enzyme catalyzes an ATP $\rightleftharpoons$ P_i exchange, there is no net synthesis of ATP, since the rate of ATP hydrolysis is faster than the rate of ATP synthesis (de Meis and Carvalho, 1974).

Recently, Knowles and Racker (1975) reported that the purified Ca^{2+}-dependent ATPase preparations from sarcoplasmic reticulum vesicles can synthesize a small amount of ATP from ADP and P_i in the absence of a calcium concentration gradient. This was achieved by a two-step procedure where initially the enzyme was phosphorylated by P_i in the presence of Mg^{2+} ions and EGTA. Subsequently, upon addi-

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[1] Abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; ATPase, adenosine triphosphatase; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; NADP, nicotinamide adenine dinucleotide phosphate.

tion of ADP and calcium ions (Ca^{2+} jump) at a concentration sufficient to saturate the low affinity site it was found that most of the phosphate of E~P was transferred to ADP forming ATP.

Previous work from this laboratory has shown that Ca^{2+} binding to the high- and low-affinity sites could be altered by changing the pH of the medium (Almeida and de Meis, 1977). Both sites exhibited an increased affinity for Ca^{2+} with high pH and a reduced affinity at lower pH values, the relative differences in affinities of the two sites remaining unchanged at all pH values studied. Based on this finding, the following rationale was developed: No ATP synthesis will be observed when the same Ca^{2+} concentration exists on each side of the membrane and at a level sufficient to saturate the high-affinity site but not sufficient to allow significant binding of Ca^{2+} to the low-affinity site. However, this system is potentially capable of synthesizing ATP if the Ca^{2+} affinity of the two binding sites is modified by varying the pH on the two sides of the membrane, alkaline inside the vesicles and acidic in the assay medium. In such a situation, the phosphorylation of the enzyme by P_i will no longer be inhibited, and the Ca^{2+} concentration could become sufficient to allow the transfer of phosphate to ADP.

In the present report this hypothesis was tested using the technique described for the Ca^{2+} jump (Knowles and Racker, 1975) except that the sudden change in Ca^{2+} concentration was substituted for by a change in pH, which should have the same ultimate effect on the binding of Ca^{2+} to the enzyme as a change in Ca^{2+} concentration.

Materials and Methods

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

Ca^{2+}-dependent ATPase was isolated from intact vesicles as described by MacLennan (1970). The solubilized enzyme was dialyzed against 0.6 M sucrose, 45 mM Tris-HCl, pH 8.0, 0.91 mM histidine, and 0.87 M ammonium acetate for 16 h and then passed through a Sephadex G-50 (medium) column (2.5 × 45 cm) equilibrated with the above solution. The fraction containing the re-formed vesicles was diluted with 1/3 volume of ice cold H_2O and centrifuged at 70 000*g* for 60 min (MacLennan et al., 1971). The pellet was suspended to a protein concentration of 13–15 mg/mL in the above solution, except that the final concentration of Tris-HCl was decreased to 5 mM. The purified enzyme was stored at −15 °C. Using the same assay medium described by MacLennan (1970), these preparations had a Ca^{2+}-dependent ATPase activity varying between 7.0 and 11.4 μmol of P_i/mg of protein·min at 37 °C.

The re-formed vesicles of Ca^{2+}-dependent ATPase were unable to accumulate Ca^{2+} under any conditions studied, even when oxalate or phosphate was included in the medium (MacLennan et al., 1971).

$^{32}P_i$ and [γ-^{32}P]ATP. $^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy and was purified by extraction as phosphomolybdate with 2-butanol–benzene, reextraction to the aqueous phase with ammonium hydroxide solution, and finally precipitated as the $MgNH_4PO_4$ salt (Kanazawa and Boyer, 1973). The $^{32}P_i$ was stored in a dilute HCl solution until used. [γ-^{32}P]ATP was prepared as described previously (de Meis, 1972).

Fast Kinetics of Phosphoenzyme Dephosphorylation and ATP Synthesis. Quenched-flow measurements were performed using a multispeed transmission device, Model no. 600.000 (Harvard Apparatus Co., Inc., Dover, Mass.) with reaction times ranging from 8 ms to 2.3 s. The linearity of the velocity of injection of the syringes was confirmed by monitoring the voltage drop across a potentiometer connected to a Tektronix Type R564B storage oscilloscope. Syringe A contained 20 mM Tris–maleate, 2 mM EGTA, 20 mM $MgCl_2$, 8 mM $^{32}P_i$ (ca. 4×10^7 cpm/μmol) and 1 mg/mL of Ca^{2+}-dependent ATPase. The final pH of this phosphorylating solution was 6.0. Syringe B contained 40 mM $CaCl_2$, 4 mM ADP, 10 mM glucose, and 4 units/mL of hexokinase (EC 2.7.1.1). Equal volumes (about 1 mL) of solutions from syringes A and B were force mixed together through a capillary tube. One end of this tube was connected to the syringes by means of a "U" junction. The other end was immersed in 2 mL of the quenching solution (250 mM perchloric acid). Different reaction times were obtained by varying both the length of the capillary tube and the flow rate of injection. The reaction was performed at room temperature (23–25 °C). The total volume of each quenched solution was measured and then centrifuged at 3000*g* for 10 min. The phosphoenzyme level and ATP formed were determined on the pellet and supernatant, respectively, as described below. The level of phosphoenzyme present in syringe A was determined at the beginning and the end of the procedure by quenching with perchloric acid an aliquot of the phosphorylating medium contained in syringe A.

For reaction times longer than 2.3 s, the device described above was not used. In these experiments, 0.5 mL of medium B was squirted with a syringe into a test tube containing 0.5 mL of medium A under intense stirring. The reaction was arrested at the appropriate time by adding 1 mL of ice-cold 250 mM perchloric acid solution. Each incubation was carried out in duplicate.

ATP Synthesis. ATP synthesis was determined by two different methods: (1) measuring the incorporation of $^{32}P_i$ into [γ-^{32}P]ATP or glucose 6-[^{32}P]phosphate, the excess $^{32}P_i$ being extracted from the assay medium as phosphomolybdate with 2-butanol–benzene (de Meis and Carvalho, 1974); (2) measuring the ATP present enzymatically. In the experiments of Table I, the ATP was isolated from the reaction medium and measured enzymatically using hexokinase and glucose-6-phosphate dehydrogenase (EC 1.1.1.4.9). In order to follow the recovery of ATP in each experiment, a tracer amount of [γ-^{32}P]ATP was added to 44 mL of the mercuric chloride quenched medium. The denatured enzyme was removed by centrifugation and the clear supernatant was added to a column (0.8 × 5.0 cm) of Dowex 1-X10-400. The column was previously washed with 20 mL of 1 N HCl, water until the eluate was at neutral pH, 20 mL of 1 N NaOH, and again with water until the eluate was at neutral pH. After the addition of the material, the column was slowly eluted with 30 mL of water, 40 mL of a solution containing 20 mM NH_4Cl, and 20 mM HCl, 30 mL of water, and finally with 5 mL of 1 N HCl. This last fraction was collected in ice. The subsequent steps were performed in the cold. Practically all of the ADP and P_i were eluted by the mixture of NH_4Cl and HCl. The ATP (55–65%) was eluted in the 5 mL of 1 N HCl. The ATP was removed from the HCl solution by precipitation as the barium salt at alkaline pH. Since the amount of ATP was very small, a carrier amount of P_i was added. To the 5-mL fraction of 1 N HCl, 0.02 mL of 0.2 M P_i solution and 0.35 mL of 0.85 M $BaCl_2$ solution were added. After mixing, the medium was made alkaline by the addition of 2.2 mL of 3 M KOH solution. The faint precipitate which formed was collected by centrifugation at 5000*g* for 10 min. The supernatant was removed, the wall of the tube dried with filter paper and the precipitate was solubilized by the addition of 0.025 to 0.035 mL of 1 N

TABLE I: Net Synthesis of ATP.[a]

Jump		ATP in the sample (μmol × 10^{-9})				Net synthesis	
			Control				
pH	$CaCl_2$ (mM)	Test	Minus P_i	Minus Ca^{2+}	Minus enzyme	Total (test–control)	μmol/g of protein
6.0 to 6.0	0 to 20.0	194	61			133	3.26
6.0 to 6.0	0 to 20.0	253	109	99		144	4.20
6.0 to 6.0	0 to 20.0	192	51			141	3.21
6.0 to 6.0	0 to 20.0	208		102		106	2.44
6.0 to 6.0	0 to 20.0	172			33	139	3.51
6.0 to 6.0	0 to 20.0	192			25	167	3.75
6.0 to 7.8	0 to 1.5	110	40			70	1.99
6.1 to 8.1	0 to 1.0	88		52		36	1.00
6.1 to 8.2	0 to 1.5	108		53		55	1.50
5.1 to 7.6	0.6 to 0.6	67	42			25	0.70
5.1 to 7.7	0.6 to 0.6	69	55			14	0.36
5.1 to 7.8	0.6 to 0.6	74		56		18	0.52
5.1 to 7.9	0.6 to 0.6	75		33		42	1.19
5.1 to 8.1	0.6 to 0.6	48		34		14	0.34

[a] The reaction was performed at room temperature using the fast-kinetic technique described under Materials and Methods. The reaction time was 300 ms. For the experiments of the Ca^{2+} jump at pH 6.0, syringe A contained 22 mL of the phosphorylation medium with 20 mM Tris-maleate buffer, 1 mM EGTA, 8 mM P_i, 20 mM $MgCl_2$, and 35 to 42 mg of Ca^{2+}-dependent ATPase protein. The final pH of the mixture was 6.0. Syringe B contained 22 mL of a solution of 40 mM $CaCl_2$ and 4 mM ADP. For the experiments of Ca^{2+} and pH jump, syringe A contained 22 mL of a solution of 8 mM P_i, 20 mM $MgCl_2$, 1 mM EGTA, and 35 to 41 mg of Ca^{2+}-dependent ATPase protein. The pH of this mixture was in the range of 6.0 to 6.1. Syringe B contained 22 mL of a solution with 2 mM ADP, 3 to 4 mM $CaCl_2$, and 21 to 23 mM KOH. For the experiment of pH jump in the presence of 0.6 mM $CaCl_2$, syringe A contained 22 mL of a solution of 13 mM maleic acid, 10 mM P_i, 20 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 35 to 41 mg of Ca^{2+}-dependent ATPase protein. The pH of the mixture was 5.1. Syringe B contained 22 mL of solution with 0.6 mM $CaCl_2$, 2 mM ADP, and 45 to 60 mM KOH. In all the experiments, the Ca^{2+}-dependent ATPase was added to the solution contained in syringe A 2 to 3 min before mixing the content of syringes A and B. The reaction was arrested with 3.5 mL of 10 mM $HgCl_2$. The ATP of this mixture was isolated, concentrated, and measured as described under Materials and Methods. In the table, *test* refers to the complete system and *control* to a parallel experiment where either P_i, Ca^{2+}, or the enzyme was omitted from the reaction media. The omitted reactant was added to the mixture after quenching with $HgCl_2$.

HCl. The barium was than precipitated as $BaSO_4$ by the addition of 0.65 mL of 30 mM $MgSO_4$. After stirring and centrifugation, the clear supernatant containing $MgSO_4$ and MgATP was taken for enzymatic assay. At this step, 20–30% of the original [γ-^{32}P]ATP was recovered.

The enzymatic assay of ATP was performed at room temperature by mixing in a cuvette 0.7 mL of the above ATP solution with 0.3 mL of a solution containing 20 mM EGTA, 200 mM Tris-HCl buffer (pH 8.5), 20 mM glucose, and 0.55 mM NADP, followed by the addition of 0.02 mL of glucose-6-phosphate dehydrogenase (82 units/mL) and 0.02 mL of hexokinase (160 units/mL). The change in optical absorbance was followed at 340 nm using a DB-GT Beckman spectrophotometer. When there was no further change in absorbance, 0.02 mL of 1 mM ATP solution was added in order to correlate the change in absorbance with the actual amount of ATP present in the mixture.

Phosphorylation by P_i. The conditions for enzyme phosphorylation were as described in the legends of figures. E~^{32}P present in the protein precipitates was washed four times with 4-mL aliquots of ice-cold 125 mM perchloric acid containing 2 mM P_i. The washed pellet was resuspended in 0.3 mL of a solution containing 0.1 N NaOH, 2% Na_2CO_3, and 1 mM P_i, and dissolved by heating in boiling water for 20 min. An aliquot of this solution was taken for liquid scintillation counting of ^{32}P and another was taken for protein determination by the method of Lowry et al. (1951). In order to measure the nonspecific binding of $^{32}P_i$, controls were performed in which Cl_3AcOH was included in the assay medium before the addition of the enzyme. All phosphorylation data were corrected for this nonspecific binding. The degree of phosphorylation varied significantly in the different preparations tested. Therefore, with the exception of the experiments presented in Figure 4, each group of experiments was performed using enzyme preparations having similar phosphoenzyme levels.

ADP Treatment. Commercial ADP preparations contain significant amounts of ATP. This could be assayed by using hexokinase and glucose-6-phosphate dehydrogenase. The extent of contamination was found to be in the range of 3 to 6 mol of ATP for each 100 mol of ADP. In order to decrease the ATP contamination, a solution containing 100 mM ADP, 110 mM $MgCl_2$, and 0.05 mM $CaCl_2$ (pH 6.8) was incubated with 0.4 mg/mL of purified Ca^{2+}-dependent ATPase for 2 h at 30 °C. The reaction was stopped by heating the incubation medium for 10 s in boiling water, followed by rapid cooling in ice. The denatured enzyme was removed by centrifugation and the clear supernatant was filtered through a Millipore filter (0.45 μm). The filtrate was stored at −5 °C. This preparation of ADP was used throughout the experiments described here. Following this treatment, the amount of ATP remaining was so low that it was not possible to measure it as described above. Therefore, the remaining contamination was determined by two different methods. (1) Before starting the hydrolysis of the ATP by the ATPase, a tracer amount of [γ-^{32}P]ATP was introduced. The level of ATP present before and after treatment was determined by measuring the amount of radioactivity that was not extracted with 2-butanol–benzene followed addition of molybdate. (2) The remaining ATP was isolated from the ADP solution and concentrated 50 times as described under ATP synthesis and then measured enzymatically. With either method it was found that the proportion of remaining ATP was 0.2–0.05 mol of ATP for each 100 mol of ADP.

Results

Inhibition of Phosphorylation by Ca^{2+}. Previous reports have shown that phosphorylation of sarcoplasmic reticulum

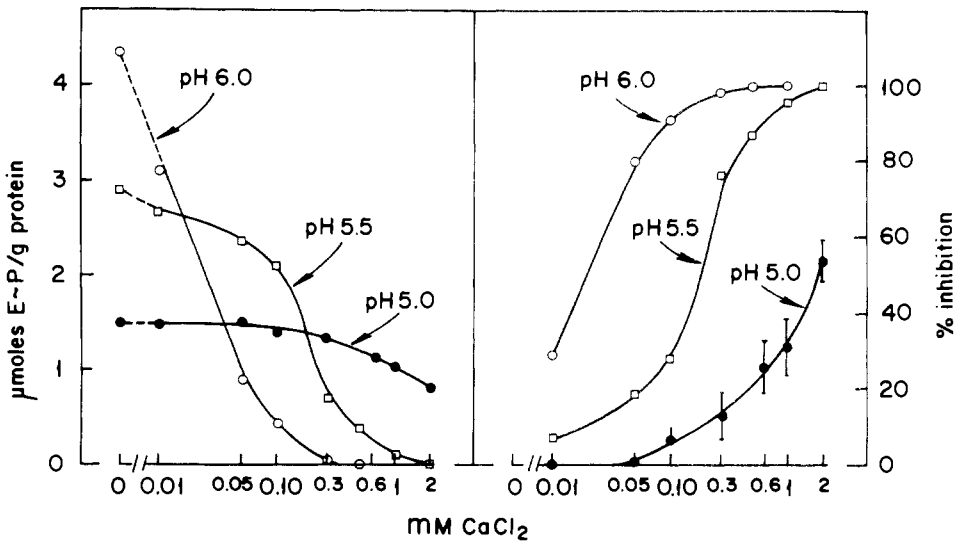


FIGURE 1: Effect of Ca^{2+} concentration and pH on the phosphorylation of the Ca^{2+}-dependent ATPase. The reaction medium contained 20 mM Tris-maleate buffer, 8 mM $^{32}P_i$ (10^7 cpm/μmol), 20 mM $MgCl_2$, 1 mg of Ca^{2+}-dependent ATPase protein, and the $CaCl_2$ concentrations shown in the figure. For 0 Ca^{2+} concentration, $CaCl_2$ was omitted and 2.5 mM EGTA was added. The final volume was 1 mL, and the pH of the mixture was 6.0 (○), 5.5 (□), and 5.0 (●). The reaction was performed at 25 °C, it was started by the addition of the enzyme, and it was arrested after 30 s of incubation by the addition of 3 mL of an ice-cold solution of perchloric acid (125 mM) containing 2 mM orthophosphate. The values represent either the average of two experiments (○, □) or the averate ± standard error of five experiments (●).

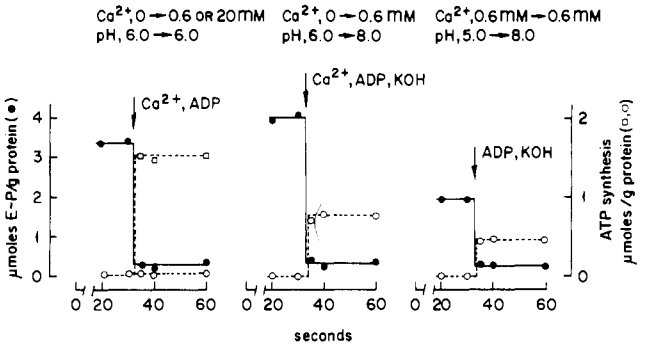


FIGURE 2: pH and Ca^{2+} jump. *Left:* The reaction medium (0.5 mL) contained 20 mM $MgCl_2$, 8 mM $^{32}P_i$ (2.5×10^7 cpm/μmol), 2 units of hexokinase, and 1 mg of Ca^{2+}-dependent ATPase protein. The pH of the medium was 6.0. The reaction was performed at 25 °C, it was started by the addition of the enzyme, and after 30 s of incubation (arrow) 0.5 mL of a solution containing 4 mM ADP, 10 mM glucose, and either 1.2 (○) or 40 mM (□) $CaCl_2$ was added. Note that the concentrations of these reagents were halved after mixing. The pH of the assay medium remained constant (6.0) after this addition. The reaction was terminated at different incubation intervals as shown in the figure by the addition of 0.1 mL of 100% Cl_3AcOH (w/v). Aliquots of the deproteinized supernatant and the protein precipitates were analyzed for [γ-^{32}P]ATP and E~^{32}P formation, respectively, as described under Methods. *Middle.* The experimental conditions and assay medium were the same as above, except that the second solution, in addition to 4 mM ADP, 10 mM glucose, and 1.2 mM $CaCl_2$ also contained 23 mM KOH. Thus, the pH of the medium increased from 6.0 to 8.0 after mixing. *Right.* The reaction medium (0.8 mL) contained 9 mM maleic acid, 20 mM $MgCl_2$, 8 mM $^{32}P_i$, 0.6 mM $CaCl_2$, 2 units of hexokinase, and 1 mg of Ca^{2+}-dependent ATPase protein. The pH was 5.0. The reaction was started by the addition of the enzyme and 30 s later 0.2 mL of a mixture of ADP, glucose, $CaCl_2$, and KOH was added to give final concentrations of 2 mM ADP, 5 mM glucose, and 40 mM KOH. The $CaCl_2$ concentration remained constant (0.6 mM). After this addition, the pH of the medium increased from 5.0 to 8.0. Other experimental conditions were as described in *left:* (○) ATP synthesis; (●) E~P.

by $^{32}P_i$ is maximal at pH 6 and at this pH is strongly inhibited by the binding of calcium at a site of high affinity located on the outer surface of the membrane (Masuda and de Meis, 1973; Kanazawa, 1975; de Meis, 1976). Figure 1 shows that when phosphorylation was performed at pH values lower than 6, although the level of E~P in the presence of EGTA decreased, the inhibitory effect of Ca^{2+} was markedly reduced. At pH 6.0, 0.6 mM $CaCl_2$ completely inhibited phosphorylation, whereas the same $CaCl_2$ concentration produced only 20 to 30% inhibition at pH 5.0.

ATP Synthesis. At pH 6.0, the phosphoenzyme formed in the presence of EGTA was able to transfer about 50% of its phosphate to ADP upon the sudden addition of 2 mM ADP and 20 mM $CaCl_2$ (final concentrations) to the assay medium (Figure 2, left). If the amount of $CaCl_2$ introduced in the assay medium was decreased to a final concentration of 0.6 mM, the phosphoenzyme was completely hydrolyzed and none of its phosphate was transferred to ADP. The reason for this finding is given by the data of Figure 3 which shows that at pH 6.0 it was necessary to have at least 2 mM $CaCl_2$ before any significant ATP synthesis could be detected. As the Ca^{2+} concentration was increased above 2 mM the amount of ATP synthesis increased and had not reached saturation even at 20–40 mM. Higher Ca^{2+} concentrations were avoided in order to prevent precipitation in the medium of calcium phosphate.

The $CaCl_2$ concentration required for ATP synthesis was found to be highly dependent upon the pH of the medium (Figures 2 and 3). This was detected by introducing KOH in the assay medium together with ADP and $CaCl_2$. Thus Figure 2 (middle) shows that ATP synthesis could be measured in the presence of 0.6 mM $CaCl_2$ if at the time of ADP and $CaCl_2$ addition the pH of the assay medium was increased from 6.0 to 8.0. Figure 3 shows that the $CaCl_2$ concentration required for half-maximal transfer of phosphate from E~P to ADP was higher than 6 mM at pH 6.0, but decreased to 2 and 0.4 mM when the pH of the medium was increased to 7.4 and 8.1, respectively.

ATP Synthesis at Constant Ca^{2+} Concentration. In Figure 1 it was shown that a significant phosphorylation of the enzyme could be measured in the presence of 0.6 mM $CaCl_2$ when the reaction was performed at pH 5.0. Figure 3 shows that at pH 8.1, this same $CaCl_2$ concentration was sufficient to allow the transfer of phosphate from E~P to ADP. Therefore, experiments were performed where E~P was formed at pH 5.0 in the presence of 0.6 mM $CaCl_2$ and subsequently dephosphorylated by adding ADP and a KOH concentration sufficient to increase the pH of the medium from 5.0 to 8.0. Figure 2 (right)

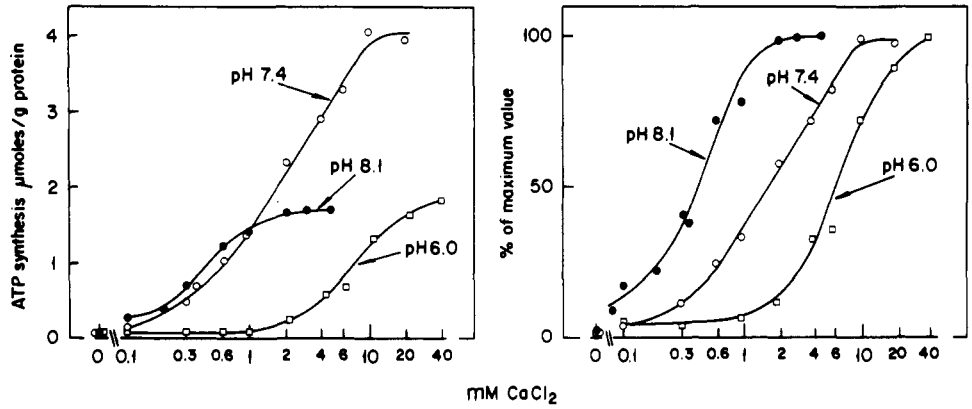


FIGURE 3: Ca^{2+} dependence. The reaction media (0.5 mL) contained 20 mM $MgCl_2$, 8 mM $^{32}P_i$, 2 units of hexokinase, and 1 mg of Ca^{2+}-dependent ATPase. The pH of the mixture was 6.0. The reaction was started by the addition of Ca^{2+}-dependent ATPase and after 30 s it was added to 0.5 mL of a solution containing 10 mM glucose, 4 mM ADP, various concentrations of $CaCl_2$ to give the final concentrations shown in the figure, and KOH in a concentration sufficient to increase the pH of the final mixture from 6.0 to either 7.4 (○) or 8.1 (●). KOH was not added to those incubations which were maintained at pH 6.0 (□). The reaction was stopped 1 min later by the addition of 0.1 mL of 100% Cl_3AcOH (w/v). Other experimental conditions were as described in Figure 2. Each point represents the average of two experiments.

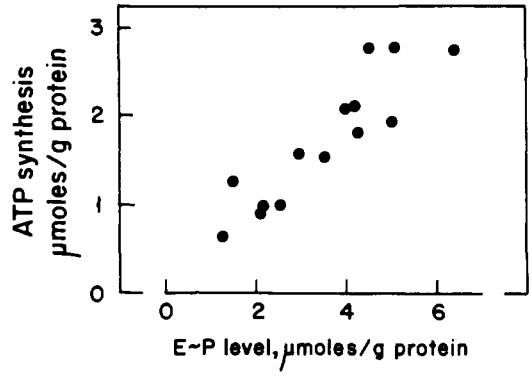


FIGURE 4: Relationship between ATP synthesis and E~P level. Data was taken from experiments where there was a large variation in the level of phosphoenzyme formation. Experimental conditions were as described in the legend to Figure 2 (left) but with a fixed final concentration of 20 mM Ca^{2+}.

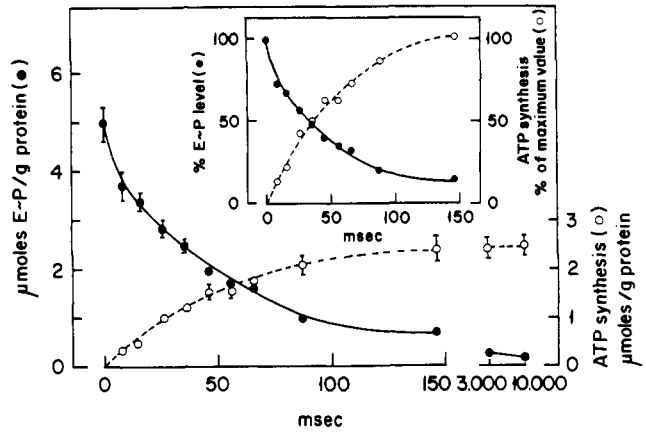


FIGURE 5: Dephosphorylation of E~P and ATP formation. The reaction medium and experimental technique were as described under Materials and Methods for fast-kinetic studies. Each point represents the mean ± standard error of three experiments. The *inset* shows the same data but expressed either as a percent of the E~P level or as a percent of maximum ATP synthesized.

shows that a small amount of ATP was synthesized under these conditions. No significant synthesis of ATP could be measured in control experiments where $CaCl_2$ was omitted from the assay medium.

ADP Dependence. Half-maximal synthesis of ATP was obtained in the ADP concentration range of 0.9 to 1.5 mM when the reaction was performed at pH 6.0 and in the range of 0.2 to 0.4 mM when performed at pH 8.1 (data not shown).

Relationship between ATP Synthesis and Initial Phosphoenzyme Level. Figure 4 shows a plot of ATP synthesis at pH 6.0 vs. the phosphoenzyme level as measured prior to the addition of 20 mM Ca^{2+} and 2 mM ADP. This plot shows that the amount of ATP synthesized depends on the original level of phosphoenzyme present. The amount of phosphate transferred from E~P to ADP after the addition of saturating concentrations of $CaCl_2$ and ADP varied with the final pH of the medium. In Figure 3, the initial steady-state level of E~P was in the range of 3.5 to 4.5 μmol/g of protein. At pH 7.4, practically all the phosphate of E~P was transferred to ADP. However, at pH 6.0 or 8.1, only 40 to 50% of the E~P phosphate was transferred. When the pH was raised from 6.0 to 9.0, no ATP synthesis could be measured upon the addition of ADP and $CaCl_2$ to a final concentration of 2 and 5 mM, respectively (data not shown).

Fast Kinetics of E~P Dephosphorylation and ATP Synthesis at pH 6.0. In order to ascertain whether the disappearance of phosphoenzyme and the synthesis of ATP were synchronous, the time course of the reactions was followed over the millisecond scale using a rapid-quenching device (Figure 5). Dephosphorylation of E~P occurred very rapidly and was almost complete within 150 ms after adding Ca^{2+} and ADP. Synthesis of ATP followed the same time course as for dephosphorylation. The time for half-maximal E~P dephosphorylation and half-maximal ATP synthesis was in the range of 30 to 40 ms. The rapid dephosphorylation of E~P was only observed when both ADP and a high concentration of Ca^{2+} were added. In the initial 150 ms practically no dephosphorylation of E~P was observed when either Ca^{2+} alone (0.1 or 20 mM) or ADP alone (2 mM) was added to the medium (Figure 6). Upon the single addition of Ca^{2+} (20 mM final), the half-time of the dephosphorylation was in the range of 2 to 4 s, i.e., about two orders of magnitude greater than that measured upon the simultaneous addition of Ca^{2+} and ADP.

Separate Addition of Ca^{2+} and ADP. In the experiments performed above, ATP synthesis was observed when Ca^{2+} and ADP were simultaneously added to the phosphorylated enzyme. However, while the Ca^{2+}-dependent ATPase vesicles

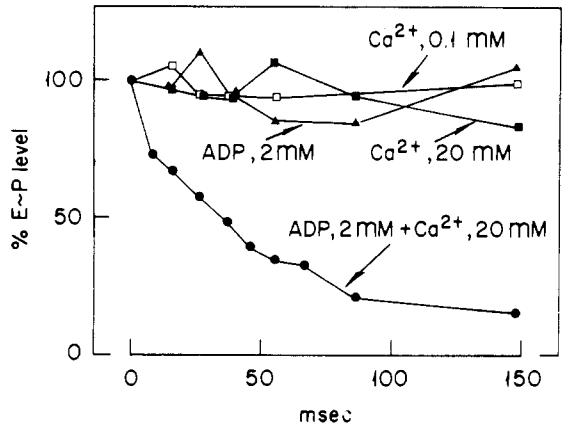


FIGURE 6: Dephosphorylation of E~P in the presence of ADP and Ca^{2+}. The reaction medium and experimental technique were as described under Materials and Methods for fast-kinetic studies, except syringe B contained either 0.2 mM $CaCl_2$ (□), 40 mM $CaCl_2$ (■), 4 mM ADP (▲), or 40 mM $CaCl_2$ plus 4 mM ADP (●). Note that the concentrations of $CaCl_2$ and ADP are halved after mixing with the contents of syringe A. Each point represents the mean of either two or three experiments.

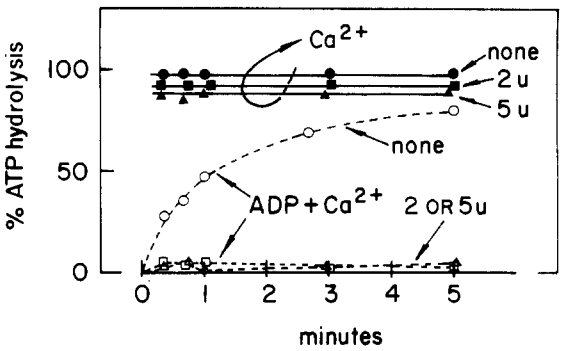


FIGURE 7: ATP hydrolysis. The reaction was performed at 23 °C by mixing equal volumes (0.5 mL) of media A and B. Media A contained 8 mM P_i, 20 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mg of Ca^{2+}-dependent ATPase and no hexokinase (○, ●), and 2 (□, ■) or 5 units (△, ▲) of hexokinase. Media B contained 5 μM [γ-^{32}P]ATP, 10 mM glucose, and no ADP (closed symbols, solid lines) or 4 mM ADP (open symbols, dashed lines). The final pH was 8.0. The reaction was stopped at various times by the addition of 0.1 mL of 100% Cl_3AcOH (w/v). The $^{32}P_i$ released was extracted with 2-butanol–benzene as described under Materials and Methods.

are leaky and unable to accumulate Ca^{2+}, the possibility existed that, upon the addition of Ca^{2+} to the medium, a transient Ca^{2+} gradient was formed across the membrane where the concentration outside was higher than in the vesicle lumen. Should the re-formed vesicles have enzymatic units arranged "outside-in", these units might utilize energy from the transient Ca^{2+} gradient for the synthesis of ATP. Since it was observed that when Ca^{2+} was added alone to the phosphorylated enzyme only a slow dephosphorylation occurred, experiments were performed at pH 6.0 where ADP was added to the medium 1 to 2 s after the addition of Ca^{2+} in an attempt to give the Ca^{2+} time to distribute itself across the membrane prior to the addition of ADP. In the six experiments performed in this manner, ATP was synthesized and the level was similar to that found in Figure 2 (left) (data not shown).

Release of ATP. In order to ascertain whether or not the ATP synthesized remained bound to the enzyme, paired experiments were performed where the reaction was arrested either by the addition of Cl_3AcOH or by filtering the mixture through Millipore filters with an average pore size of 0.45 μm. The assay media composition and the experimental conditions were the same as those described in Figure 2, left (20 mM $CaCl_2$). The amount of [γ-^{32}P]ATP found in the Cl_3AcOH treated sample and in the filtrate was essentially the same, indicating that the ATP synthesized was released from the enzyme into the medium. If ATP remained bound to the enzyme, one would not expect to find [γ-^{32}P]ATP in the filtrate. Similar results were reported by Knowles and Racker (1975).

ATP Hydrolysis. In order to avoid the hydrolysis of the synthesized ATP in the experiments of Figures 2 to 5, glucose and hexokinase were added to the assay medium. Figure 7 shows a control experiment where the rate of hydrolysis of the synthesized ATP at pH 8.0 and the ability of hexokinase to trap the synthesized ATP were measured. The experimental conditions used were similar to those described in Figure 2 (middle) using nonradioactive P_i. An amount of [γ-^{32}P]ATP similar to that expected to be formed from E~P and ADP was added to the assay medium together with ADP, $CaCl_2$, and KOH.

In the presence of both ADP and $CaCl_2$, about 50% of the added [γ-^{32}P]ATP was hydrolyzed in the first minute of incubation. This could be avoided by the addition of glucose and hexokinase which trapped practically all the ATP added as glucose 6-phosphate. When ADP was omitted from the reaction medium, the hydrolysis proceeded at a very fast rate and hexokinase was unable to trap the added ATP. Essentially the same results were obtained when this experiment was performed at pH 6.0 under the conditions of Figure 2 (left).

ATP Contamination. When ADP and high concentrations of Ca^{2+} are added to the phosphorylating medium, the final mixture has an ideal composition for the enzyme to catalyze an ATP⇌P_i exchange reaction. During this exchange, the enzyme simultaneously hydrolyzes and synthesizes ATP, where the rate of hydrolysis is several fold faster than the rate of synthesis (de Meis and Carvalho, 1974; Carvalho et al., 1976). Therefore, if a significant amount of ATP is introduced into the system, the appearance of ^{32}P in the water phase after 2-butanol–benzene extraction might not necessarily indicate that there is net synthesis of ATP. ATP contamination might arise from either the addition of commercial ADP preparations or indirectly from contamination of the ATPase preparation with adenylate kinase which will form ATP and AMP from added ADP. Prior to its purification by the MacLennan procedure (1970), the sarcoplasmic reticulum vesicles are contaminated with adenylate kinase (Beirão and de Meis, 1976). The experiment described in Figure 2 was repeated, but using leaky vesicles contaminated with adenylate kinase. Figure 8 illustrates how such conditions can give misleading information. During the initial step, Ca^{2+}-dependent ATPase was phosphorylated by $^{32}P_i$, forming about 1 μmol of E~P per g of protein. Upon the addition of Ca^{2+} and ADP, the enzyme was immediately dephosphorylated, and a large incorporation of $^{32}P_i$ into the aqueous phase occurred. Not only did the incorporation of $^{32}P_i$ far exceed the amount of E~P dephosphorylated but also the time course of the ^{32}P incorporation was completely different from that of the dephosphorylation.

Further confirmation that the [γ-^{32}P]ATP measured in Figure 8 was derived from an ATP⇌P_i exchange and did not reflect a net synthesis of ATP was obtained from another set of experiments using leaky vesicles under conditions identical to those of Figure 8. Five minutes after the addition of ADP and Ca^{2+} to the phosphorylating medium, the reaction mixture was filtered through a Millipore filter (0.45 μm) and the ATP content in the filtrate was determined enzymatically using hexokinase and glucose-6-phosphate dehydrogenase as described under Materials and Methods. A control was performed omitting P_i from the phosphorylating medium. In all

experiments, a larger amount of ATP was found in the control (without P_i) than in the complete system. The actual amount of ATP measured was in the micromolar range and varied between different vesicle preparations tested (data not shown).

Net Synthesis of ATP. In order to confirm that, in the experiments described in Figures 2 to 5, the radioactivity detected in the water phase after extraction with 2-butanol–benzene indeed reflected a net synthesis of ATP, a series of experiments was performed using large volumes of assay medium. The assay medium composition and experimental conditions were similar to those described in Figure 2, except that glucose and hexokinase were omitted. The fast kinetic device was used in order to achieve a good mixing of the large volumes used and to avoid the hydrolysis of the ATP synthesized. After arresting the reaction, the ATP contained in the mixture was isolated, concentrated, and assayed enzymatically as described under Materials and Methods. Table I shows that in all experiments performed the amount of ATP measured in the complete system was clearly higher than that measured in the control experiments. The ATP isolated from the control experiments represents a summation of the ATP contaminating the ADP solution (see Materials and Methods), the tracer [γ-^{32}P]ATP added for determining the recovery of ATP during the purification, and any ATP which might have been formed by traces of adenylate kinase which might still contaminate the Ca^{2+}-dependent ATPase.

The following considerations leads to the conclusion that in Figures 2 to 5 and in Table I the synthesis of ATP was catalyzed by the Ca^{2+}-dependent ATPase and not by adenylate kinase.

(1) The synthesis of ATP was proportional to the amount of phosphoenzyme (Figure 4) and the total amount of ATP formed never exceeded the initial amount of E~P formed. As shown in Figure 8, if the synthesis of ATP was promoted by adenylate kinase the amount of ATP formed would largely exceed the initial level of E~P.

(2) The rate of ATP synthesis was equivalent to the rate of E~P disappearance (Figure 5). After dephosphorylation of E~P, there was no more synthesis of ATP. If the synthesis of ATP was promoted by adenylate kinase, this correlation would not be found. Figure 8 shows that in presence of adenylate kinase the synthesis of ATP continued for several minutes after the dephosphorylation of E~P.

(3) In the experiments of Table I, controls were performed in which either P_i or Ca^{2+} was omitted from the assay media. These ions are not required for adenylate kinase activity (Beirão and de Meis, 1976). Therefore, one should find the same amount of ATP in the "control" and "test" if the synthesis of ATP was catalyzed by contaminating adenylate kinase. Table I clearly shows that this was not the case.

Discussion

The experiments reported were performed with leaky vesicles reconstituted from purified Ca^{2+}-dependent ATPase. These vesicles were unable to accumulate Ca^{2+}. In the following discussion these data will be extended to sealed vesicles. The Ca^{2+}-binding sites of high and low affinity will be taken as references to identify the outer and inner surface of the membrane.

Reaction Sequence. On the basis of accumulated evidence (de Meis and de Mello, 1973; Masuda and de Meis, 1973; de Meis and Masuda, 1974; de Meis and Carvalho, 1974, 1976; Ikemoto, 1974, 1975, 1976; de Meis and Sorenson, 1975; Kanazawa, 1975; Knowles and Racker, 1975; de Meis, 1976; Souza and de Meis, 1976) the following reaction sequence was recently proposed (Carvalho et al., 1976).

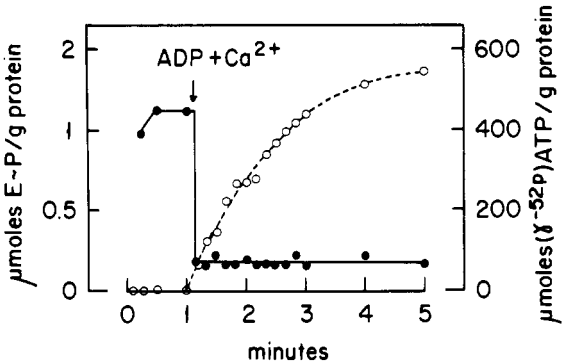
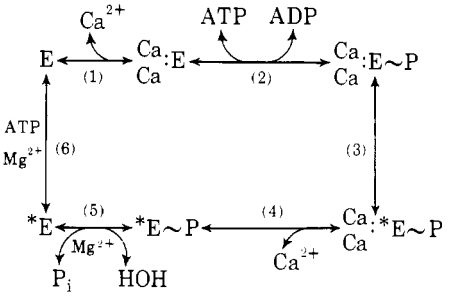


FIGURE 8: Phosphorylation and [γ-^{32}P]ATP synthesis in leaky vesicles contaminated with adenylate kinase. The reaction medium (0.5 mL) contained 10 mM Tris–maleate buffer (pH 6.0), 2 mM EGTA, 10 mM $MgCl_2$, 4 mM $^{32}P_i$ (1×10^7 cpm/μmol), and 1 mg of leaky vesicles. One minute after the addition of leaky vesicles, 0.1 mL of a medium containing 30 mM Ca^{2+}, 30 mM ADP, 18 mM glucose, and 20 units of hexokinase was added (arrow). The reaction was performed at 26 °C. At various times, up to 5 min, the reaction was stopped with 0.05 mL of 100% Cl_3AcOH. Aliquots of the deproteinized supernatant were analyzed for [γ-^{32}P]ATP and the protein precipitates were analyzed for E~^{32}P formation as described under Materials and Methods.



In this reaction sequence, the Ca^{2+}-dependent ATPase is represented in two different conformations, E and *E. In the E form, the site which translocates Ca^{2+} through the membrane faces outwards from the surface of the membrane and has a high affinity for Ca^{2+} (apparent K_m at pH 7.0, 1–3 μM); E can be phosphorylated by ATP but not by P_i. In the *E conformation, the Ca^{2+}-binding site faces the vesicle lumen and has a low affinity for Ca^{2+} (apparent K_m at pH 7.0, 1–3 mM). *E can be phosphorylated by P_i but not by ATP. The interconversion of *E into E (reaction 6) is the slowest reaction of the sequence. ATP, besides phosphorylating the enzyme, can also activate the rate of interconversion of *E to E.

The Reversibility of Reactions. The synthesis of ATP is initiated by the phosphorylation of *E by P_i (reaction 5). The reversibility of reaction 5 has been demonstrated by measuring the disappearance of phosphoenzyme when the P_i concentration of the medium is decreased (Masuda and de Meis, 1973), and by measuring the rate of $P_i \rightleftharpoons HOH$ exchange (Kanazawa and Boyer, 1973; Boyer et al., 1977). This exchange represents a dynamic reversal of reaction 5. The rate of interchange of phosphate between P_i of the medium and *E~P under steady-state conditions is very rapid, the half-time for 50% substitution of the *E~P phosphate being in the range of 30 to 50 ms (Boyer et al., 1977). Both the phosphorylation by P_i and the $P_i \rightleftharpoons HOH$ exchange occur in the absence of a transmembrane Ca^{2+} concentration gradient (Masuda and de Meis, 1973; Boyer et al., 1977).

Phosphorylation of the enzyme by P_i requires that the outer surface of the vesicle membrane be exposed to a medium where

the Ca^{2+} concentration is very low. If the Ca^{2+}-binding site of high affinity is saturated (reaction 1), *E will be converted slowly into E (reaction 6) and the P_i-unreactive form $\overset{Ca}{Ca}E$ will be accumulated. The data presented in this paper show that the half-time of E~P decomposition through reactions 5, 6, and 1 is in the range of 2 to 4 s.

The data of Figure 3 shows that the phosphate of E~P is only transferred to ADP (reactions 4, 3, and 2) if Ca^{2+} binds to the site of low affinity (reaction 4). In Figure 2, when Ca^{2+} was introduced in the assay medium, the Ca^{2+}-binding site of high affinity was saturated. However, net synthesis of ATP was still observed because the rate by which *E~P transfers its phosphate to ADP is about 100 times faster than the rate of *E~P hydrolysis and transformation into E. In previous reports (de Meis and Carvalho, 1974; de Meis and Sorenson, 1975), it was shown that, in the experimental conditions of Figure 2, reactions 4, 3, and 2 will flow back and forth if ATP is also introduced in the assay medium (ATP $\rightleftharpoons$ P_i exchange). Therefore, these reactions are also reversible and occur in the absence of a Ca^{2+} concentration gradient.

Ca^{2+} Concentration Gradient and Energy Transduction. From the data discussed above it is concluded that each of the individual reactions involved in the synthesis of ATP is reversible and can flow forward or backward without the need for utilization of the energy which might be derived from the concentration gradient of Ca^{2+} across the membrane, i.e., from the electrochemical potential of Ca^{2+}. In the presence of Mg^{2+}, the rate of either ATP synthesis or ATP hydrolysis will depend on the asymmetrical binding of calcium on the two sides of the membrane and on the relative concentrations of P_i, ADP, and ATP in the medium. When calcium binds only to the site of low affinity, reaction 1 becomes irreversible and ADP plus P_i will operate as driving forces which would move the reactions in the direction of ATP synthesis. When calcium binds only to the site of high affinity, reaction 4 becomes irreversible and ATP will move the reaction in the direction of ATP hydrolysis. When calcium binds to both sites, the enzyme will simultaneously catalyze the hydrolysis and the synthesis of ATP (ATP $\rightleftharpoons$ P_i exchange). In this condition the ratio between the hydrolysis and synthesis of ATP will depend on the relative concentrations of ATP, ADP, and P_i in the medium (Carvalho et al., 1976). Therefore, for the synthesis of ATP in sealed vesicles, the larger difference of Ca^{2+} concentrations on the two sides of the membrane is solely needed to meet the differences of affinities of the external and internal Ca^{2+}-binding sites. This conclusion is further supported by the data of Figure 2 and Table I, where it is shown that a modification of the Ca^{2+} affinity of the two binding sites induced by a modification of the pH of the assay medium (pH jump) can promote the synthesis of ATP under conditions where the Ca^{2+} concentration is maintained constant.

At present we do not know from which source the energy is derived for the synthesis of ATP. This might be derived from the binding of substrates and ions to the enzyme as has been suggested for the (Na^+,K^+)ATPase (Taniguchi and Post, 1975; Post et al., 1975; Knowles and Racker, 1975; Kuriki et al., 1976).

Proton Gradient. According to the data of Figures 1 to 3, when the Ca^{2+} concentration on both sides of the membrane of a sealed vesicle is 0.6 mM, the Ca^{2+}-dependent ATPase is only able to drive the synthesis of ATP if the pH in the assay medium is 5.0 and inside the vesicle in the range of 7.5 to 8.0. In this condition there is a transmembrane H^+ concentration gradient and not a Ca^{2+} gradient. This finding indicates a new mechanism by which ATP can be synthesized when there is a transmembrane proton gradient. This model does not require an ATPase specifically designed for the transport of protons (Mitchell, 1974; Boyer, 1975; Williams, 1975). A different transport ATPase such as the Ca^{2+}-dependent ATPase may be used and the synthesis of ATP occurs without the need to use the energy which might be derived from the movement of protons through the membrane, i.e., from the electrochemical potential of H^+ (Mitchell, 1974). The difference of pH on the two sides of the membrane is solely required to modify the affinity of the two Ca^{2+}-binding sites of the enzyme.

It is possible that other transport ATPases can also drive the synthesis of ATP by a similar mechanism. For instance, it has been recently shown that the Na^+,K^+-dependent ATPase can also synthesize a small amount of ATP from ADP and P_i in the absence of a transmembrane concentration gradient of either K^+ or Na^+ (Taniguchi and Post, 1975; Post et al., 1975; Kuriki et al., 1976). Both the phosphorylation of the enzyme by P_i and the transfer of phosphate from E~P to ADP depend on the asymmetrical binding of K^+ and Na^+ on the two sides of the membrane. If the affinity of the Na^+ and K^+ binding sites also varies with the pH of the medium, then the (Na^+,K^+)ATPase is also potentially capable of driving the synthesis of ATP when a H^+ gradient is formed. Therefore, several different transport ATPases could be mobilized for the synthesis of ATP whenever there is a difference of pH on the two sides of a membrane, even when there is no difference in the concentration of the ion regularly transported by these ATPases.

Summation of Ca^{2+} and H^+ Gradient. According to the data of Figure 2 (left) when the pH on both sides of the membrane is 6.0 there is no synthesis of ATP when a sealed vesicle containing 0.6 mM $CaCl_2$ is incubated in a medium containing EGTA. Although the enzyme is phosphorylated by P_i (Figure 1), the Ca^{2+} concentration inside the vesicle will not be sufficient to activate the transfer of phosphate from E~P to ADP (Figure 3). But if in addition to this small difference of Ca^{2+} concentration there is also a difference of pH (1.5–2 units), then the system becomes capable of driving the synthesis of ATP (Figure 2, middle). Figure 3 (right) and Table I show that when the Ca^{2+} concentrations on the two sides of the membrane are equal (0.6 mM) it is necessary to have a larger pH difference (2.5–3 units) in order to have ATP synthesis. These data indicate that there is a reciprocal counterbalance of H^+ and Ca^{2+} concentration to reach the critical asymmetrical binding of Ca^{2+} on the two sides of the membrane. A small difference in concentration of one ionic species (Ca^{2+} or H^+) can be compensated for by a small difference in concentration of the other ionic species, neither one alone being able, in isolation, to drive the synthesis of ATP.

Ca^{2+} and H^+ Translocation. Makinose and Hasselbach (1971) have shown that, coupled with the synthesis of one ATP molecule, two calcium ions are released by sarcoplasmic reticulum vesicles. The experimental approach used in this report does not permit measurement of whether or not in the conditions of Figure 2 (middle and left) there is also an H^+ translocation coupled with the synthesis of ATP. The following consideration might, however, indicate a theoretical possibility where the synthesis of ATP is coupled with the simultaneous ejection of Ca^{2+} to the assay medium and accumulation of H^+ in the vesicle lumen.

Evidence has been presented that the binding of Ca^{2+} on the two sides of the membrane is related to a single site on the Ca^{2+}-dependent ATPase which translocates Ca^{2+} through the membrane (de Meis and Carvalho, 1974; de Meis and Sorenson, 1975; Ikemoto, 1975, 1976).

The affinity of this site for Ca^{2+} increases with the increase of pH of the medium. This is likely to be related to the presence of a negatively charged proton-accepting group such as a

carboxyl located in the Ca^{2+}-binding site. Therefore, when facing the vesicle lumen (alkaline), the group would lose H^+ and, simultaneously, Ca^{2+} would bind to the site. After translocation, the site would face the outer surface (acidic) where Ca^{2+} would be released and the group would gain H^+. A similar mechanism of proton translocation has been recently proposed by Boyer (1975). The difference in this particular case is that the proton-accepting radical would reside within the same Ca^{2+}-binding-site region of the Ca^{2+}-dependent ATPase molecule.

Final Remarks. The above considerations are not intended to imply a model system to be applied to mitochondria or to a physiological role of pH in skeletal muscle, but simply to contribute to our understanding of the mechanism by which a concentration gradient of an ion can be utilized for the synthesis of ATP by biological membranes other than that of mitochondria. The architecture of the energy-transducing ATPase of mitochondria is quite different from that of the Ca^{2+}-dependent ATPase. At present, there is no evidence that under physiological conditions the two sides of the sarcoplasmic reticulum membrane are exposed to media with different pH.

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