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Role of the Ca^{2+} Concentration Gradient in the Adenosine 5′-Triphosphate–Inorganic Phosphate Exchange Catalyzed by Sarcoplasmic Reticulum†

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ABSTRACT: Sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle catalyze a Ca^{2+}-dependent exchange between orthophosphate and the γ-phosphate of ATP. This exchange occurs both in the presence and in the absence of a transmembrane Ca^{2+} gradient. The exchange catalyzed by solubilized or leaky sarcoplasmic reticulum vesicles is activated by ADP, Ca^{2+} (K_m = 1.6–2.4 mM), and orthophosphate (K_m = 38 mM). In contrast, when a Ca^{2+} concentration gradient is formed across the membrane of intact vesicles, the K_m of orthophosphate is only 3.2 mM. The Ca^{2+} concentration required for half-maximal activation of the ATP $\rightleftharpoons$ P_i exchange reaction is 10^3–2×10^4 times higher than that required for half-maximal activation of Ca^{2+} transport and Ca^{2+}-dependent ATP hydrolysis. In the presence of 8 mM Ca^{2+}, the ATPase activity of leaky or solubilized vesicles is 20–150 times higher than that of ATP $\rightleftharpoons$ P_i exchange.

A highly efficient ATP-dependent system for Ca^{2+} transport has been described in SRV[1] isolated from skeletal muscle (Hasselbach, 1964). In the process of ATP hydrolysis the γ-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 (Yamamoto and Tonomura, 1967; Makinose, 1969; de Meis, 1972). The following sequence has therefore been proposed.

$$\text{ATP} + \text{E} \underset{Mg^{2+}}{\overset{Ca^{2+}}{\rightleftharpoons}} \text{E}-\text{P} + \text{ADP} \quad (1)$$

$$\text{E}-\text{P} \overset{Mg^{2+}}{\rightleftharpoons} \text{E} + \text{P}_i \quad (2)$$

Recently it has been shown that these two reactions can be reversed, *i.e.*, that the Ca^{2+} pump of the SRV is reversible (Barlogie *et al.*, 1971; Makinose, 1971, 1972, 1973; Makinose and Hasselbach, 1971; Hasselbach *et al.*, 1972; Panet and Selinger, 1972; Deamer and Baskin, 1972; Masuda and de Meis, 1974). The following data support this finding.

(*a*) *NET Synthesis of ATP.* When SRV previously loaded with calcium phosphate are incubated in a medium containing ADP, Mg^{2+}, and $^{32}P_i$, it is observed that $^{32}P_i$ interacts with the membrane forming E–P, Ca^{2+} is released at a very fast rate, and [γ-$^{32}P_i$]ATP is formed (Barlogie *et al.*, 1971; Makinose, 1972, 1973; Makinose and Hasselbach, 1971).

(*b*) *ATP $\rightleftharpoons$ P_i Exchange.* When SRV are incubated in a medium containing ATP, Mg^{2+}, $^{32}P_i$, and Ca^{2+}, calcium phosphate is accumulated by the vesicles and a Ca^{2+} concentration gradient is built up until a steady state is reached in which a slow Ca^{2+} efflux is balanced by an ATP-driven influx. When this condition is reached, a steady rate of exchange between P_i and the γ-phosphate of ATP is observed. It has been implied that this exchange is the result of the two reactions shown above operating simultaneously forward (ATP hydrolysis) and backward (ATP synthesis from ADP and $^{32}P_i$) (Makinose, 1971; Racker, 1972). If the SRV are made "leaky" by means of phospholipase A or ether, although the transport ATPase remains unimpaired (Makinose, 1971), the Ca^{2+} concentration gradient is abolished and the ATP $\rightleftharpoons$ P_i exchange reaction is arrested. These data lend support to Mitchell's chemiosmotic hypothesis (conversion of osmotic into chemical energy). Accordingly, the energy required for E–P formation, ATP synthe-

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‡ Recipient of a fellowship from the Conselho Nacional de Pesquisas, Brazil.

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

sis, and ATP $\rightleftharpoons$ P_i exchange would be derived from the steep Ca^{2+} concentration formed across the SRV membrane (Makinose, 1971, 1972, 1973; Makinose and Hasselbach, 1971; Mitchell, 1966; Mitchell and Moyle, 1967).

In a previous paper (Masuda and de Meis, 1973; de Meis and Masuda, 1974) it has been shown that $^{32}P_i$ can react with the SRV membrane forming a high energy E-P in the absence of a Ca^{2+} concentration gradient. Similar results were reported by Kanazawa and Boyer (1973). These authors also observed that SRV catalyze a rapid $P_i \rightleftharpoons$ HOH exchange independently of a Ca^{2+} concentration gradient. Recently, Racker (1972) reported that the ATP $\rightleftharpoons$ P_i exchange reaction could be catalyzed by solubilized SRV.

In this paper the ATP $\rightleftharpoons$ P_i exchange reaction and its correlation with the Ca^{2+} concentration gradient were further investigated.

Material and Methods

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

[γ-^{32}P]ATP was prepared as previously described (de Meis, 1972).

$^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy and purified by means of a column of Dowex AG1-X10 resin (200–400 mesh) previously treated with 1 N NaOH. After the addition of the $^{32}P_i$ solution, the column was washed with deionized water and the $^{32}P_i$ was eluted with 0.04 N HCl. The $^{32}P_i$ was stored in a 0.04 N HCl solution until used.

ATP $\rightleftharpoons$ P_i exchange was assayed by measuring the formation of [γ-^{32}P]ATP from ADP and $^{32}P_i$. The excess of $^{32}P_i$ was removed by the method of Avron (1960), slightly modified. A 0.1-ml aliquot of the assay medium was mixed with 0.15 ml of trichloroacetic acid (10%, w/v) and 0.15 ml of acetone. After 5-min incubation in ice, 0.2 ml of a 2.5 N H_2SO_4 solution containing 5% (w/v) ammonium molybdate was added, followed by 1.5 ml of a mixture of isobutyl alcohol and benzene (1:1, v/v). After 5-min incubation at room temperature, the tube was vigorously stirred for 40 sec. After phase separation, the isobutyl alcohol–benzene layer was removed and 0.02 ml of 20 mM KH_2PO_4 was added to the aqueous layer. The extraction with isobutyl alcohol–benzene was repeated and after removal of the organic phase an aliquot of the water phase was counted in a liquid scintillation counter. After this extraction only 0.01–0.05% of the original content of $^{32}P_i$ remained in the aqueous phase. This was negligible in the standard assay. As a control, after extraction by the Avron method, the [γ-^{32}P]ATP formed in the different experimental conditions described in the results was identified by paper chromatography, by chromatography on a Dowex AG1-X10 resin (de Meis, 1972), and enzymatically (Panet and Selinger, 1972) with the use of hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.4.9).

ATPase activity was assayed by measuring the release of $^{32}P_i$ from [γ-^{32}P]ATP. The $^{32}P_i$ was extracted from the assay medium by the method of Avron as described above, except that after the first extraction with isobutyl alcohol–benzene the tube was centrifuged 3 min at 5000*g* and an aliquot of the isobutyl alcohol–benzene phase was counted in a liquid scintillation counter.

Preparation of Leaky SRV. A suspension of SRV at 9 mg of protein/ml in 1 mM EGTA was adjusted to pH 9.2 by the addition of 1 M Tris. After 20-min incubation at room temperature the pH was readjusted to 7.0 by the addition of small amounts of crystalline maleic acid (Duggan and Martonosi, 1970), and the suspension was used immediately. This treatment increases the Ca^{2+} permeability of the SRV membrane, allowing any Ca^{2+} which might have accumulated inside the vesicles to flow out. In other experiments, the SRV were rendered leaky by the addition to the assay medium of 40 μM X-537-A, an ionophore that facilitates passive diffusion of Ca^{2+}, therefore preventing accumulation by the SR pump. As a control, Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity were measured before and after this treatment. In agreement with the reports of other authors (Inesi *et al.*, 1967; Fiehn and Hasselbach, 1969; Duggan and Martonosi, 1970) the leaky vesicles no longer accumulated Ca^{2+} although the ATPase activity remained unimpaired (Table I).

TABLE I: Correlation between SRV Membrane Permeability and ATP $\rightleftharpoons$ P_i Exchange.[a]

SRV Treatment	% Ca^{2+} Bound	ATP $\rightleftharpoons$ P_i (μmol/g of Protein per 5 min)	ATPase Act. (μmol P_i/mg of Protein per 5 min)
None	98.8 ± 1.3	380.30 ± 42.50	2.26 ± 0.65
X-537-A, 40 μM	0	1.28 ± 0.97	12.44 ± 1.49
2 mM EGTA, pH 9.2	0	3.12 ± 1.98	12.86 ± 1.70
Solubilization with Triton X-100		0	14.53 ± 0.75

[a] The assay medium contained 30 mM Tris-maleate buffer (pH 6.8), 20 mM $MgCl_2$, 0.1 mM $CaCl_2$, 6 mM potassium phosphate buffer (pH 6.8), and 5 mM ATP (intact SRV) or 10 mM ATP (leaky or solubilized SRV). The reaction was started by the addition of SRV to a concentration of 0.3 mg/ml and stopped after 5-min incubation at 37° by the addition of Cl_3CCOOH to a final concentration of 6% (w/v). The values represent the average ± standard error of five experiments.

Ca^{2+} uptake was assayed by the use of ^{45}Ca and Millipore filters as previously described (de Meis, 1969, 1971).

Solubilized SRV was prepared as described by Ikemoto *et al.* (1971). SRV were suspended at a final concentration of 10 mg/ml, in a medium containing 50 mM Tris-HCl buffer (pH 8.0) and 20% (v/v) glycerol. Triton X-100 was added slowly, while stirring, to a final concentration of 20–25 mg/ml, followed by $CaCl_2$ to a final concentration of 4 mM. This mixture was stored overnight at 5° and centrifuged at 105,000*g* for 90 min. The clear supernatant was stored at 5° and used within a period of 3 days.

Results

Ca^{2+} Uptake and ATP $\rightleftharpoons$ P_i Exchange. Orthophosphate activates SRV Ca^{2+} uptake through the precipitation of calcium phosphate within the vesicles (Hasselbach, 1972; de Meis *et al.*, 1974). Figure 1 shows that the rate of Ca^{2+} uptake is progressively enhanced when higher concentrations of P_i are added to the assay medium. During the initial 30–60 sec of incubation, when only 20–30% of the Ca^{2+} content of the medium had been removed by the vesicles, little or no ATP $\rightleftharpoons$ P_i exchange could be measured. This

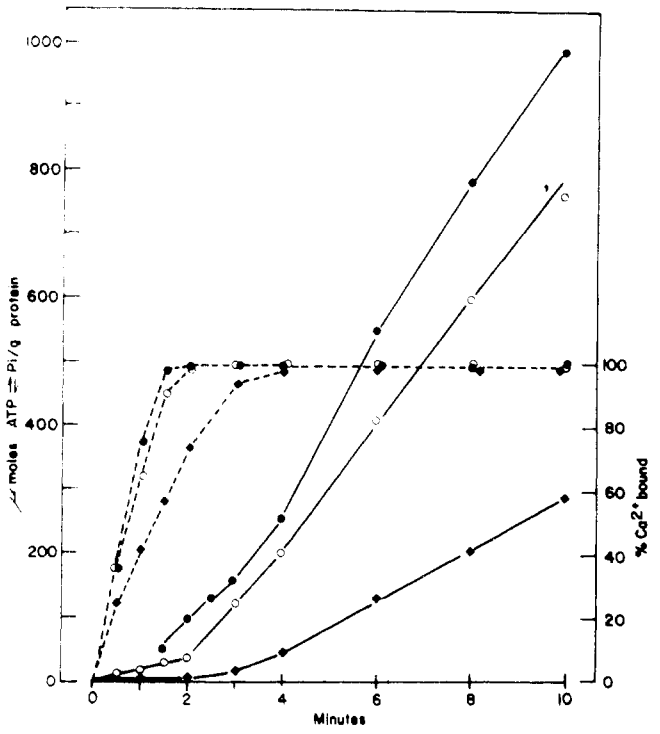

FIGURE 1: Rate of ATP $\rightleftharpoons$ P_i exchange and of Ca^{2+} uptake. The assay medium composition was 20 mM Tris-maleate buffer (pH 6.8), 4 mM ATP, 15 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mg/ml of SRV protein, and (♦) 4 mM, (○) 7 mM, or (●) 10 mM potassium phosphate (pH 6.8). The reaction was performed at 30°. It was started by the addition of SRV and stopped after different incubation intervals by removal of SRV with Millipore filters. For Ca^{2+} uptake (dashed lines) nonradioactive P_i and ^{45}Ca were used. For ATP $\rightleftharpoons$ P_i exchange (full lines), $^{32}P_i$ and nonradioactive Ca were used.

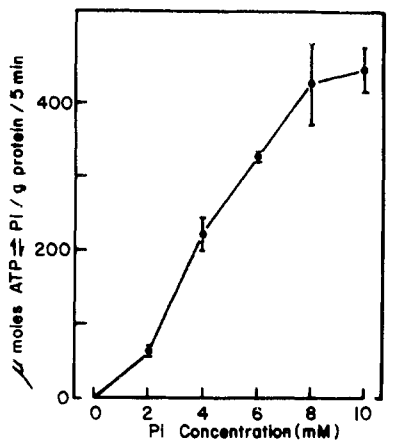

FIGURE 2: P_i dependence. The experimental conditions and the assay medium were the same as in Figure 1. The reaction was performed at 37°. The rate of ATP $\rightleftharpoons$ P_i exchange was measured after 99% of the Ca^{2+} of the assay medium had been removed by the SRV. The values shown in the figure represent the average ± standard error of four experiments.

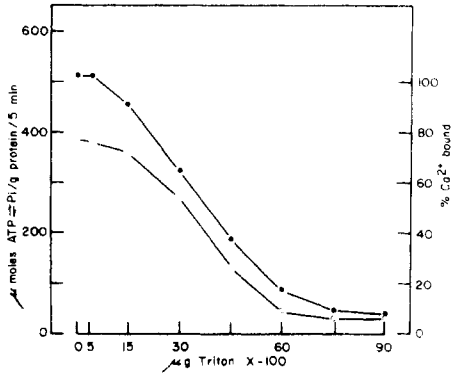

FIGURE 3: Correlation between Ca^{2+} uptake and ATP $\rightleftharpoons$ P_i exchange. The assay medium composition was 20 mM Tris-maleate buffer (pH 6.8), 4 mM ATP, 10 mM potassium phosphate (pH 6.8), 12.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM EGTA, and 0.2 mg/ml of SRV protein. The reaction was performed at 30°. For Ca^{2+} uptake (○) ^{45}Ca was used. For ATP $\rightleftharpoons$ P_i exchange (●), $^{32}P_i$ was used.

rate was progressively enhanced with prolonged incubation intervals, reaching a maximum when practically all the Ca^{2+} of the medium had been accumulated by the SRV. The dependence of the ATP $\rightleftharpoons$ P_i exchange on Ca^{2+} accumulation by the SRV could be observed more clearly when the rate of Ca^{2+} uptake was decreased by the use of low concentrations of P_i. These observations are in agreement with the data reported by Makinose (1971). The data of Figures 1 and 2 show that after all the Ca^{2+} of the medium is removed by the vesicles, the rate of ATP $\rightleftharpoons$ P_i exchange varies with the P_i concentration of the assay medium. Plotting the data of Figure 2 as the reciprocal of the initial velocity of ATP $\rightleftharpoons$ P_i exchange *vs.* the reciprocal of the square of the concentration of P_i yields a straight line whose intercept gives an apparent K_m for P_i varying between 4 and 6 mM. This value is essentially the same as the K_m for P_i reported for the reversal of the Ca^{2+} pump (Masuda and de Meis, 1974). Figure 3 shows that when the SRV were made leaky by the addition to the incubation medium of minute concentrations of Triton X-100, a parallel decrease of both the Ca^{2+} accumulating capacity and of the rate of ATP $\rightleftharpoons$ P_i exchange was observed. Table I shows that with a Ca^{2+} concentration of 0.1 mM in the assay medium, the rate of ATP $\rightleftharpoons$ P_i exchange was practically abolished when the SRV were solubilized or rendered leaky by different experimental procedures.

Correlation between Ca^{2+} Concentration and ATP $\rightleftharpoons$ P_i Exchange. The data shown above can lead to the conclusion that the ATP $\rightleftharpoons$ P_i exchange reaction demands the formation of a Ca^{2+} concentration gradient across the membrane. On the other hand, when this gradient is formed, the inner face of the SRV membrane is exposed to a high Ca^{2+} concentration. The aim of the following set of experiments, using solubilized or leaky vesicles, was to ascertain whether the simple exposure of the membrane to a high Ca^{2+} concentration would be sufficient to activate the ATP $\rightleftharpoons$ P_i exchange reaction or whether this reaction is only activated when a Ca^{2+} concentration gradient is formed across the membrane.

Figure 4 shows that the ATPase activity of solubilized or leaky vesicles was progressively inhibited when the Ca^{2+} concentration of the medium was raised from 0.3 to 10 mM. No ATP $\rightleftharpoons$ P_i exchange could be measured in the presence of 0.1 mM Ca^{2+}. However, raising the Ca^{2+} concentration of the medium resulted in a progressive activation of the ATP $\rightleftharpoons$ P_i exchange reaction, reaching a maximum in the Ca^{2+} concentration range of 4–5 mM. In 11 different preparations tested, the Ca^{2+} concentration required for 50% of maximum activation was found to vary between 1.6 and 2.3 mM.

Effect of pH. In the presence of 3 mM $CaCl_2$, maximal activation of the ATP $\rightleftharpoons$ P_i exchange was observed at pH 7.0, whereas for the ATPase activity activation was maximal in the pH range of 6.1–6.4. In these experiments the P_i concentration was decreased to 1 mM in order to avoid the

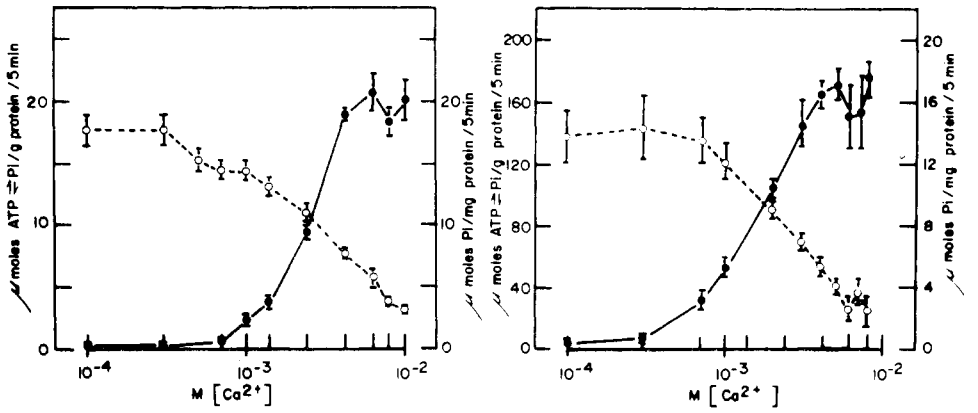

FIGURE 4: Ca^{2+} dependence of ATP $\rightleftharpoons$ P_i exchange. The assay medium composition was 30 mM Tris-maleate buffer (pH 6.8), 10 mM ATP, 6 mM $^{32}P_i$, 20 mM $MgCl_2$, and 0.3 mg/ml of SRV protein. For the ATPase activity (O- - -O), [γ - ^{32}P]ATP was used. For ATP $\rightleftharpoons$ P_i exchange (●-●), $^{32}P_i$ was used. The reaction was performed at 37°. Other conditions are as described under Methods: (left) solubilized SRV; (right) leaky SRV (2 mM EGTA, pH 9.2). The values shown in the figure represent the average ± standard error of five experiments.

precipitation of Ca^{2+} phosphate in the assay medium in the alkaline range of the pH curve.

ADP Dependence. The data of Figure 5 show that ADP is required as a substrate for the ATP $\rightleftharpoons$ P_i exchange. The formation of [γ-^{32}P]ATP is arrested if an ATP-regenerating system is included in the medium in order to avoid the accumulation of ADP derived from the ATPase activity. The addition of increasing concentrations of ADP (2-12 mM) to the assay medium resulted in no significant change in the rate of ATP $\rightleftharpoons$ P_i exchange. These data show that the ADP derived from the ATPase activity was sufficient to maximally activate the rate of [γ-^{32}P]ATP formation. Therefore, we could not measure the K_m of ADP for this reaction.

P_i Dependence. Figure 6 shows that in leaky vesicles and with a high Ca^{2+} concentration in the medium, the rate of ATP $\rightleftharpoons$ P_i exchange varies with the P_i concentration of the assay medium. Similar results were found using solubilized SRV. Table II shows the values of K_m and V_m found for the different preparations tested.

Mg^{2+} Dependence. Magnesium is required as an activating ion for the ATP $\rightleftharpoons$ P_i exchange reaction. In an assay medium containing 4 mM ATP, 4 mM $CaCl_2$, and 6-10 mM P_i, the $MgCl_2$ concentration required for maximal activation was in the range of 10-20 mM for solubilized SRV and 30-40 mM for leaky SRV.

Ca^{2+} Concentration Gradient. Table III shows that, in the presence of 6 mM $^{32}P_i$, the rate of ATP $\rightleftharpoons$ P_i exchange varies significantly depending on whether intact, leaky, or solubilized SRV were used. Figure 7 shows that the rate of ATP $\rightleftharpoons$ P_i exchange was essentially identical for leaky SRV and intact SRV previously loaded with calcium phosphate, in a medium containing 6 mM $^{32}P_i$ and 8 mM Ca^{2+}. These concentrations were very close to those required for the precipitation of calcium phosphate. A 20% increase of the concentration of either Ca^{2+} or $^{32}P_i$ resulted in the precipitation of calcium phosphate in the medium. Therefore, for the untreated SRV, the Ca^{2+} concentration on either side of the membrane should be quite similar, *i.e.*, there should be little or no Ca^{2+} concentration gradient across the membrane. On the other hand, the rate of ATP $\rightleftharpoons$ P_i exchange was significantly higher when SRV loaded with calcium phosphate were kept in a medium containing a low Ca^{2+} concentration. These data show that the rate of ATP $\rightleftharpoons$ P_i exchange is enhanced by the concentration gradient. The data of Table II indicate that this activating effect is related to an increase of the affinity of the SRV membrane for P_i.

Effect of Mitochondrial Inhibitors. The rate of ATP $\rightleftharpoons$ P_i exchange catalyzed by leaky SRV was not modified upon the addition to the assay medium of the following substances: 2 mM sodium azide, 2 mM potassium cyanide, 0.5 mM 2,4-dinitrophenol, or 20 mM AMP. In these experiments, the assay medium composition was the same as that

TABLE II: K_m of P_i for the ATP $\rightleftharpoons$ P_i Exchange Reaction.[a]

SRV Treatment	K_m (mM)	V_m (μmol of ATP $\rightleftharpoons$ P_i/g of Protein per 5 min)
None	3.17 ± 0.42	640 ± 50
2 mM EGTA, pH 9.2	38.75 ± 7.74	710 ± 120
Solubilization with Triton X-100	37.33 ± 8.97	136 ± 28

[a] The values of K_m and V_m were calculated from the data of Figure 2 (untreated SRV), Figure 6 (leaky SRV), and from experiments similar to those of Figure 6 for solubilized SRV. The values represent the average ± standard error of four experiments.

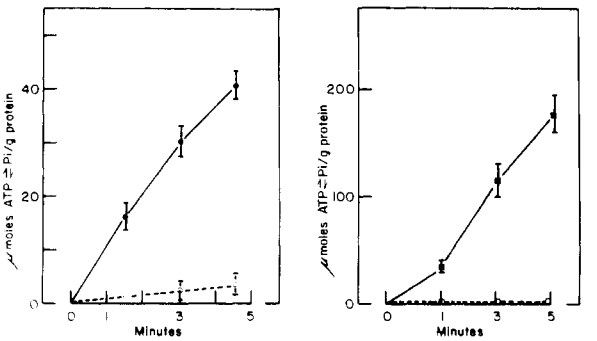

FIGURE 5: ADP dependence. The assay medium composition was 30 mM Tris-maleate buffer (pH 6.8), 10 mM ATP, 10 mM (left) or 6 mM (right) $^{32}P_i$, 4 mM $CaCl_2$, 20 mM $MgCl_2$, and 0.3 mg/ml of solubilized SRV (left) or leaky SRV (right). The reaction was performed at 37°: (●-●) and (■-■), control without ATP-regenerating system; (O- - -O) and (□- - -□), with 20 mM phosphoenolpyruvate and 200 μg/ml of ATP:pyruvate phosphotransferase (EC 2.7.1.40). The values shown in the figure represent the average ± standard error of three experiments.

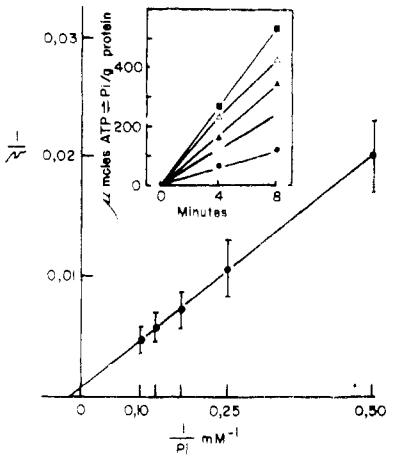

FIGURE 6: P_i dependence. The assay medium composition was 30 mM Tris-maleate buffer (pH 6.8), 10 mM ATP, 20 mM $MgCl_2$, 4 mM $CaCl_2$, 0.4 mg/ml of leaky SRV protein, and in the inset (●) 2 mM, (○) 4 mM, (▲) 6 mM, (△) 8 mM, or (■) 10 mM $^{32}P_i$. The reaction was performed at 37°. The SRV were rendered leaky by incubating them at pH 9.2 in the presence of 2 mM EGTA as described under Methods. The values shown in the figure represent the average ± standard error of four experiments.

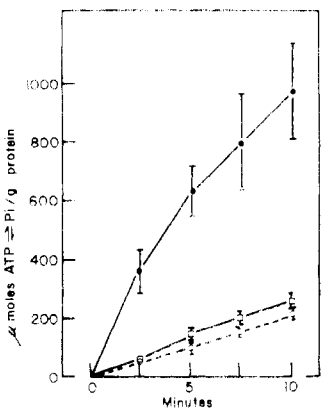

FIGURE 7: The role of the Ca^{2+} concentration gradient. The basic assay medium composition was 30 mM Tris-maleate buffer (pH 6.8), 20 mM $MgCl_2$, 6 mM $^{32}P_i$, and 10 mM ATP. The reaction was performed at 37°: (●–●) 0.3 mg/ml of SRV protein was incubated in the basic assay medium containing in addition 0.1 mM $CaCl_2$; the rate of ATP ⇌ P_i exchange was measured after 98% of the Ca^{2+} of the assay medium had been removed by the SRV; (○- - -○) same as (●–●) but after the vesicles had been loaded, the Ca^{2+} concentration of the medium was raised to 8 mM; (□–□) 0.3 mg/ml of leaky SRV protein was incubated in the basic assay medium containing in addition 8 mM $CaCl_2$. The SRV were rendered leaky (pH 9.2, 2 mM EGTA) as described under Methods. The values shown in the figure represent the average ± standard error of three experiments.

described in Figure 7.

Net Synthesis of ATP. In several SRV preparations, we failed to measure any significant ATP synthesis upon incubation of leaky SRV (0.5 mg/ml) in a medium containing 30 mM Tris-maleate buffer (pH 7.0), 4 mM $CaCl_2$, 25 mM $MgCl_2$, 8 mM $^{32}P_i$, 2 mM ADP, 20 mM AMP, 100 mM glucose, and 100 μg/ml of hexokinase. Traces of myokinase (EC 2.7.4.3) were found to contaminate our SRV preparations. Therefore, AMP was included in the assay medium in order to inhibit the synthesis of ATP from ADP that is catalyzed by myokinase.

Discussion

Role of the Ca^{2+} Concentration Gradient. The data presented show that the ATP ⇌ P_i exchange reaction is catalyzed by the SRV in the absence of a Ca^{2+} concentration gradient provided that the membrane is exposed to a high Ca^{2+} concentration, *i.e.*, that the conditions inside the SRV after Ca^{2+} accumulation are reproduced. The data of Figure 7 and Table III show that the rate of exchange is enhanced by the concentration gradient, and Table II indicates that this effect is correlated with an increase of the membrane affinity for P_i. Table II also shows that the maximal velocities of ATP ⇌ P_i exchange in intact and in leaky SRV are in the same range. In a previous paper the SRV phosphorylation by $^{32}P_i$ was studied (de Meis and Masuda, 1974). Evidence has been presented that the nucleoside triphosphate and P_i phosphorylate a common site of the membrane. The degree of membrane phosphorylation by ATP varies with the Ca^{2+} concentration of the assay medium (Yamamoto and Tonomura, 1967; Makinose, 1969; de Meis, 1972; de Meis and de Mello, 1973). In the experimental conditions of Figures 1, 2, and 7, when the maximal concentration gradient is formed, the Ca^{2+} concentration remaining in the assay medium is in the range of 1 μM (de Meis, 1971). At this Ca^{2+} concentration, only 10–25% of the membrane sites are phosphorylated by ATP (de Meis, 1972; de Meis and de Mello, 1973). Therefore, a large number of sites is left available to be phosphorylated by P_i (de Meis and Masuda, 1974). This might account for the low K_m for P_i found for both the ATP ⇌ P_i exchange and the outflux of Ca^{2+} (Masuda and de Meis, 1974). On the other hand, at a Ca^{2+} concentration in the range of 4–10 mM (Figure 4), more than 50% of the membrane sites are phosphorylated by ATP (de Meis, 1972; de Meis and de Mello, 1973). This might account for the high values of the apparent K_m for P_i found in the experiment of Figure 6. In this experiment the apparent K_m for P_i would be increased due to a competition between ATP and P_i for a common membrane site.

Upon solubilization, the original arrangement of the membrane proteins and lipids is lost, *i.e.*, the solubilized vesicles represent a much less organized system than the leaky vesicles. This might account for the decrease of the V_m of the ATP ⇌ P_i exchange reaction (Tables II and III).

Membrane Polarity. In the different models proposed for the active transport of ions it has been postulated that the affinities of the membrane for the ion transported should be different at the external and internal surfaces of the membrane. For the SRV, the external surface of the vesicle

TABLE III: Rate of ATP ⇌ P_i Exchange.[a]

SRV Treatment	$[Ca^{2+}]$ (mM)	ATP ⇌ P_i (μmol/g of Protein per 5 min)
None	0.1	444.66 ± 73.71
2 mM EGTA, pH 9.2	4.0	152.02 ± 16.09
Solubilization with Triton X-100	4.0	31.07 ± 3.61

[a] The assay medium contained 30 mM Tris-maleate buffer (pH 6.8), 20 mM $MgCl_2$, 10 mM ATP, 6 mM potassium [^{32}P]-phosphate (pH 6.8), and the $CaCl_2$ concentrations shown in the table. For the untreated SRV preparations, the rate of ATP ⇌ P_i exchange was measured after the assay medium Ca^{2+} had been taken up by the vesicles. The values represent the average ± standard error of ten experiments.

should have a high affinity for Ca^{2+} in order to be able to bind this ion even when the Ca^{2+} concentration in the assay medium is lower than 10^{-6} M. After translocation through the membrane, the affinity of the transport system for Ca^{2+} should decrease significantly in order to permit the release of the bound Ca^{2+} into the vesicle lumen (Hasselbach, 1972; Inesi, 1972; Makinose, 1973). The Ca^{2+} influx into the vesicle is promoted by the Ca^{2+}-dependent ATPase. Evidence has been presented showing that the membrane phosphorylation by ATP takes place at the external surface of the vesicle (Inesi, 1972; Hasselbach, 1972). The apparent K_m of Ca^{2+} both for the Ca^{2+}-dependent ATPase and for membrane phosphorylation has been reported to vary between 10^{-7} and 2×10^{-6} M (Inesi, 1972; Hasselbach, 1972; de Meis, 1972; de Meis and de Mello, 1973; Martonosi *et al.*, 1974). On the other hand, the active efflux of Ca^{2+} has been shown to be intimately related to the synthesis of ATP from ADP and P_i (Makinose and Hasselbach, 1971; Hasselbach, 1972; Panet and Selinger, 1972; Makinose, 1973). The data presented in this paper imply that the ATP synthesis depends on the appearance of a high Ca^{2+} concentration inside the vesicle, the K_m of Ca^{2+} being $1.6-2.4 \times 10^{-3}$ M (Figure 4). This could mean that the affinity for Ca^{2+} of the external surface of the SRV membrane is 2×10^4–10^3 times higher than that of the inner surface.

The binding of Ca^{2+} by SRV has been studied by different authors. It has been shown that SRV contain three different binding sites which can be distinguished by their respective Ca^{2+} affinities. Contradictory results have been reported on whether or not the binding of nucleoside triphosphate to the membrane modifies the association constant and binding capacity of these sites (Carvalho, 1966; Carvalho and Lee, 1967; Chevallier and Butow, 1971; MacLennan, 1970; MacLennan *et al.*, 1971; Hasselbach, 1972; Ikemoto *et al.*, 1972; Meissner, 1973; Ikemoto, 1974).

The site of higher affinity (association constant $3-10 \times 10^6$ M^{-1}) has been correlated with the membrane phosphorylation by ATP (Inesi, 1972; Hasselbach, 1972; Ikemoto, 1974). Recently, Ikemoto (1974) has presented evidence that the binding of Ca^{2+} to the site of lower affinity (association constant $1.0-1.6 \times 10^3$ M^{-1}) could be involved in the inhibition of the ATPase by excess Ca^{2+}. This site might also be involved in the ATP $\rightleftharpoons$ P_i exchange reaction.

Rate of ATP $\rightleftharpoons$ P_i Exchange and ATPase Activity. The data of Figure 4 suggest that inhibition of the ATPase activity results in an activation of ATP $\rightleftharpoons$ P_i exchange. However, in the presence of 8 mM Ca^{2+}, the rate of ATP hydrolysis was still 20 times (leaky SRV) or 150 times (solubilized SRV) higher than the rate of ATP $\rightleftharpoons$ P_i exchange. It may be that part of the energy derived from the ATPase activity is utilized for the ATP $\rightleftharpoons$ P_i exchange. This would explain why we failed to measure ATP synthesis in a system containing only ADP and P_i. However, there still remains the puzzling observation that the SRV membrane can be phosphorylated by $^{32}P_i$ to form a high energy intermediate in the absence of a Ca^{2+} concentration gradient and ATP (Kanazawa and Boyer, 1973; Boyer *et al.*, 1973; Masuda and de Meis, 1973; de Meis and Masuda, 1974).

Acknowledgment

The excellent technical assistance of Mr. Isaltino R. Soares and Mr. Valdecir Antunes Suzano is gratefully acknowledged. The antibiotic X-537A was a kind gift of Dr. Julius Berger from Hoffmann-La Roche Inc.

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Chlorotetracycline as a Fluorescent Probe for Membrane Events in the Action of Colicin K on *Escherichia coli* †

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ABSTRACT: The mechanism of action of the protein antibiotic, colicin K, was investigated with a fluorescent probe, chlorotetracycline (CT). *Escherichia coli* cells when added to medium containing CT cause an enhancement of system fluorescence. Subsequent addition of colicin K causes a twofold increase in fluorescence. This fluorescence effect of colicin K is manifested concurrently with loss of activity of the cellular galactoside transport system and with cell death. Studies with metabolic inhibitors indicate that some form of energized membrane state is necessary for colicin to act. With lactate as carbon source, anoxia prevents colicin action. In the presence of oxygen and lactate, CT fluorescence caused by colicin K is reversible by anoxia while loss of cell viability is irreversible. Characterization of the fluorescence response indicates dependence upon magnesium. Use of [^{3}H]tetracycline as a tracer shows that the colicin-stimulated fluorescence results from an increased cellular uptake of probe from the medium rather than from an increased quantum yield. Fluorescence anisotropy studies suggest that, in the absence of colicin, CT is associated with the membrane. Colicin K causes CT to fluoresce from a more fluid environment, presumably the cytoplasm of the cell. A model is presented that interprets the findings on CT fluorescence with and without colicin in terms of interactions between CT, membrane, and magnesium ions.

Colicin K, a protein antibiotic, is capable of interacting with sensitive *Escherichia coli* with kinetics consistent with a single molecule per cell being sufficient to cause death as measured by inability to form colonies. Other effects are inhibition of macromolecular syntheses [DNA, RNA, protein, polysaccharide, and lipid (Jacob *et al.*, 1952; Nomura, 1963; Nomura and Maeda, 1965)], leakiness to internal potassium (Nomura and Maeda, 1965; Hirata *et al.*, 1969; Feingold, 1970; Wendt, 1970; Dandeu *et al.*, 1969), inability to accumulate amino acids and galactosides (Fields and Luria, 1969a), and a lowering of ATP levels (Fields and Luria, 1969b). These physiological changes caused by colicin point to a generalized structural change in the membrane of the cell. Cell morphology is not noticeably changed and the cells retain their ability to concentrate substances that are transported by the phosphoenolpyruvate system (Fields and Luria, 1969a).

In this study, fluorescent probes were employed in an attempt to monitor continuously colicin-caused changes in the membrane. Light emission by these probes, when excited at a suitable wavelength, is weaker in aqueous solution and stronger in a hydrophobic environment. Cramer and Phillips (1970) have shown that colicin E1 caused a fluorescence enhancement of the probe ANS.[1] Their data demonstrated an all-or-none phenomenon, suggesting that ANS monitors a conformational change of the membrane placing the probe into a more hydrophobic environment. Phillips and Cramer (1973) found a similar colicin-caused fluorescence effect with a similar fluorescent probe, *N*-phenylnaphthylamine, and showed that the rate of colicin-caused fluorescence was dependent on both temperature and the fatty acid composition of the membrane (Cramer *et al.*, 1973).

A new fluorescent probe, chlorotetracycline (CT), was employed in the present study of the mechanism of action of colicin K. CT is bacteriostatic by its ability to bind to ribosomes and to inhibit protein synthesis (Franklin, 1963). CT fluorescence is 20 times higher when it chelates magnesium or calcium in an aqueous solvent and is 100–200 times higher in the presence of magnesium or calcium in a hydrophobic environment such as a biological membrane (Caswell and Hutchison, 1971). Caswell (1972) has used this probe to monitor changes in divalent cation distribution in the mitochondrial membrane upon energization. The data presented here suggest that colicin K, in a reaction that re-

† From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received April 22, 1974.* This investigation was supported by research grants from the National Science Foundation (GB 30575) and National Institutes of Health (AI 03038) to Dr. S. E. Luria and was conducted during the tenure of a Damon Runyon Cancer Research Fellowship.

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[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonic acid; CT, chlorotetracycline; TMG, methyl β-D-thiogalactoside; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.