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Rapid Kinetic Studies of Active Ca^{2+} Transport in Sarcoplasmic Reticulum

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Summary. A method is reported for the rapid and continuous monitoring of active Ca^{2+} transport events occurring in isolated skeletal sarcoplasmic reticulum (SR). The method is based on the quantitative evaluation of increases in the fluorescence of 1-anilino-8-naphthalenesulfonate (ANS^-), resulting from active transport. The method, which has a time resolution of 20 msec, was applied to the kinetics of Ca^{2+} transport by a Ca^{2+}-ATPase-rich SR fraction and the effects and loci of action of Mg^{2+} and monovalent cations (M^+) were investigated. The turnover number of the enzyme and its ability to establish gradients were investigated in the absence of the complicating effects of precipitating anions. The results are explicable in terms of the model of Kanazawa et al. (Kanazawa, T., Yamada, S., Yamamoto, T., Tonomura, Y., 1971, *J. Biochem. (Tokyo)* **70**:95) and are difficult to reconcile with models in which the enzyme is considered to be electrogenic. The major observations of the study are as follows:

1) Active uptake of 29 μM free Ca^{2+} in the presence of 5 mM KCl, initiated by the addition of 10^{-4} M Mg^{2+} and 2×10^{-4} M ATP, occurs with a $t_{\frac{1}{2}}$ of ca. 9 sec. The process results in an internal free Ca^{2+} concentration of 13 mM.

2) Preincubation with 50 mM KCl and 5 mM $MgCl_2$, followed by initiation of active uptake by the addition of ATP to give a final concentration of ca. 2.5 mM Mg^{2+} and ca. 2.5 mM MgATP, gave faster and larger uptakes. The $t_{\frac{1}{2}}$ for the reaction was ca. 600 msec and the internal free Ca^{2+} concentration was 70 ± 20 mM. The turnover number of 7.1 ± 0.8 sec^{-1} was calculated for the enzyme at mid-reaction under the assumption of a stoichiometry of 2 Ca^{2+} per cycle.

3) The accelerative effects of Mg^{2+} and M^+ on the rate of transport were investigated. Experiments in which the cations were added to or omitted from the incubation medium showed that the presence of both classes of activator in the internal aqueous space was necessary for maximal activation of the transport system. The concentration dependencies of these effects were investigated. Analysis shows that the monovalent cation effect is probably based on the counter-transport according to the model of Kanazawa et al. (1971) while the Mg^{2+} effect referable to the inside surface is primarily catalytic. No Mg^{2+} counter-transport could be demonstrated under conditions in which the internal monovalent cation concentration was adequate.

4) Under conditions in which the Mg^{2+} concentration is adequate for stimulation but the M^+ concentration is not (and vice versa), the active uptake can be resolved into two phases. The rapid phase is complete within the first 50 msec and corresponds to 0.26–2.06 Ca^{2+} released to the internal phase per Ca^{2+}-ATPase. These results correspond closely to those of published studies measuring the rate at which Ca^{2+} becomes inaccessible to the external solution. The comparison shows that Ca^{2+} is released to the internal aqueous phase almost as rapidly as it becomes inaccessible to the outside phase. Analysis of the concentration dependencies shows that K^+/Ca^{2+} or Mg^{2+}/Ca^{2+} competition for occupation of the inwardly-oriented translocator (of the phosphorylated enzymes) is involved in the fast phase of Ca^{2+} release. When the internal concentrations of both K^+ and Mg^{2+} are adequate, the slow phase is speeded up to such an extent that the first partial turnover can no longer be kinetically isolated from the subsequent turnovers. Under these conditions, the rate of enzyme dephosphorylation, the binding of K^+ to the translocator, and its return to an outward orientation are no longer rate limiting. The rate constant for the outward-to-inward reorientation of this translocator is ca. 13.8 sec^{-1}. The average turnover number for the first several turnovers, obtained under conditions of maximal stimulation, is ca. 7.1. The latter value

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was somewhat influenced by trans-inhibition by internal Ca^{2+}. It is concluded that this outward-to-inward transition of the Ca^{2+}-laden translocator is rate-limiting to the first turnover and that the rate of the inward-to-outward transition of the K^+-laden translocator becomes limiting in the final phases of the transport process.

5) Two major lines of evidence against electrogenic models of pump function were the stimulatory effect of internal K^+ on the transport reaction and the lack of a stimulatory effect as an inwardly-directed Cl^- gradient. Also the mechanism of the reponse of the KCl impermeable vesicles to valinomycin was investigated. Those findings also run counter to the expectations of electrogenic pump mechanisms.

The present communication makes use of 1-anilino-8-naphthalenesulfonate (ANS^-) as a kinetic indicator of Ca^{2+} transport in the ATPase-rich fraction of sarcoplasmic reticulum (SR). Use is made of the ability of ANS^- to report transport events with half-times of as short as 20 msec. The information obtained is compared with the findings of other studies which have provided a basis for understanding the cyclic mechanism by which the ATPase pumps Ca^{2+}.

A large amount of biochemical and biophysical information has been obtained about the Ca^{2+} storage and uptake function of SR (*cf.* MacLennan & Holland, 1975). Two Ca^{2+} are taken up per ATP split (Hasselbach & Makinose, 1963), and the SR pump function is able to reduce the external Ca^{2+} to submicromolar concentrations (Weber, Herz & Reiss, 1966). The uptake is sufficiently rapid to account for the rate of muscle relaxation (Inesi & Scarpa, 1972). A model of the Ca-Mg-ATPase function as an active carrier has been proposed by Kanazawa et al. (1971). The model is based on observations of the dependence of the ATPase activity and degree of phosphorylation of the enzyme upon Ca^{2+} and Mg^{2+} concentrations. It predicts that the active transport process involves the obligatory exchange of Ca^{2+} for Mg^{2+} or 2 K^+ (or other available cations). Since the publication of their studies, a number of laboratories have reported biochemical kinetic evidence for additional states of the enzyme. Synthesis of this information (*cf.* Froehlich & Taylor, 1975, 1976; Inesi et al., 1978*a*, 1978*b*; Noack et al., 1978) gives rise to the kinetic scheme presented in Fig. 1. According to this scheme, $E\sim P$ represents a covalently phos-

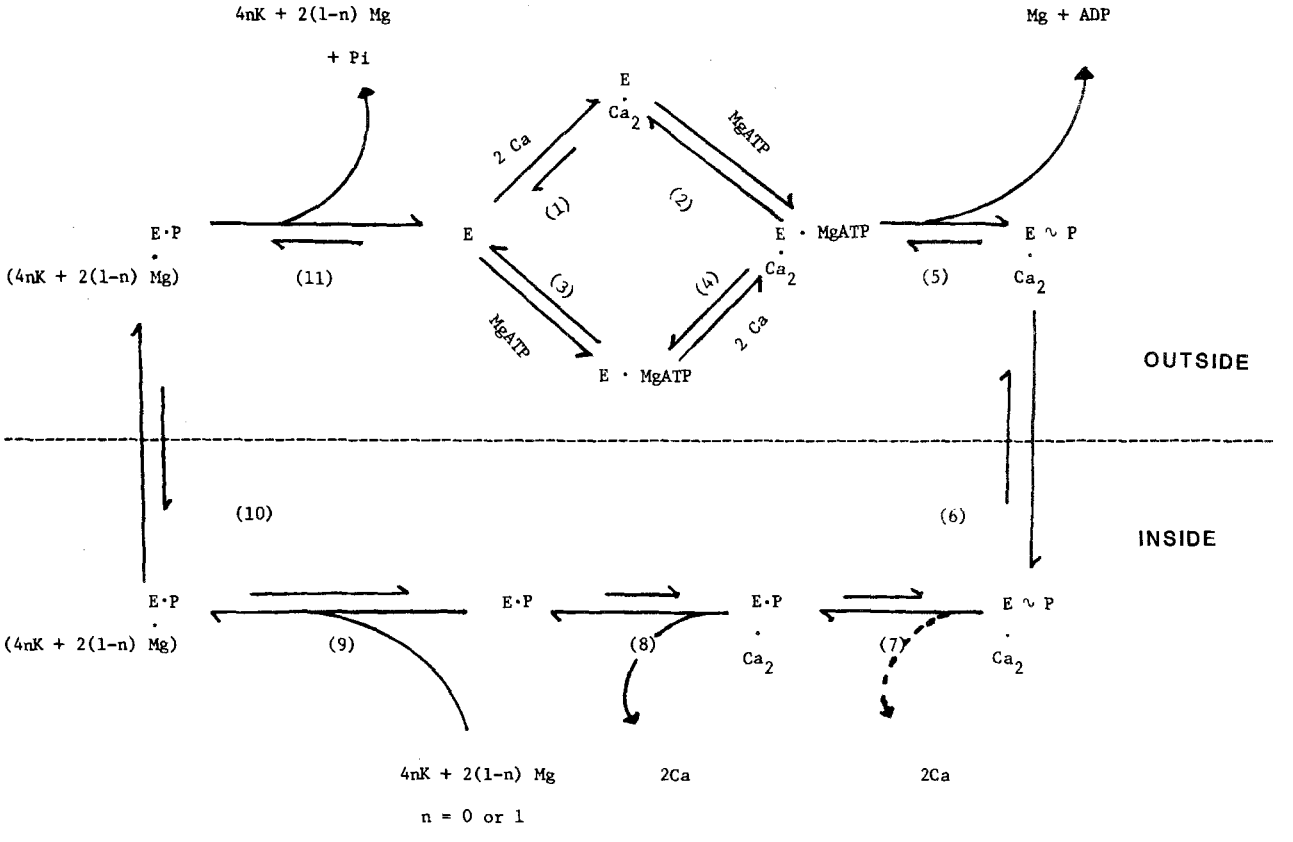

Fig. 1. Reaction scheme of ATP hydrolysis and cation transport by the SR

phorylated form of the enzyme having a high affinity for Ca^{2+} and *E.P* is a noncovalent phosphoenzyme resulting from the hydrolysis of $E\sim P$ (Froehlich & Taylor, 1975, 1976). From the requirement of active transport it can be inferred that the *E.P* state has a low affinity for Ca^{2+}. Experiments on the spin-labeled enzyme show that the inwardly-oriented Ca^{2+} binding site ($E\sim P$ or *E.P* state) has a 10^3-fold lower affinity for Ca^{2+} than does the outwardly-oriented one (Inesi et al. 1978*a, b*). In the case where K^+ or Mg^{2+} can compete with Ca^{2+} for occupation of the active site, translocation of the active site from the inside to the outside surface would result in the countertransport of these ions. In Fig. 1 the stoichiometry of the exchange is 2 Ca^{2+} for $4n$ $K^+ + 2(1\text{-}n)$ Mg^{2+} where n can be either zero or one. Studies of Yamada, Yamamoto and Tonomura (1970) showed that H^+ can be ruled out as an ion engaged in stoichiometric countertransport, although it is possible that the pump can provide a mechanism for the equilibration of H^+ (Chiu & Haynes, 1977).

The above cited studies do not provide clear evidence as to whether countertransport via the ATPase is absolutely necessary. Alternative mechanisms are shown in Fig. 2. The net transport mechanism would be the cotransport of anions via the ATPase (mechanism *II*) or could result from cotransport of anions via a separate channel in response to electrogenic Ca^{2+} transport by the ATPase (mechanism *IV*). Likewise, countertransport could be accomplished by an electrogenic Ca^{2+} pump giving rise to countertransport of cations via a cation permeability channel (mechanism *III*). These mechanisms have been analyzed for the case of passive Ca^{2+} transport via the ATPase pump in the previous studies (Haynes & Chiu, 1977, 1978; Chiu et al., 1980; Chiu & Haynes, 1980), and it was concluded that the cation countertransport mechanisms (*I* and *III*) were more rapid than anion cotransport mechanisms (*II* and *IV*).

As can be inferred from the results of these studies, the question of whether cation countertransport of anion cotransport is more important in the active transport reaction will be of great practical consequence to the evaluation of the activity of the isolated pump.

The countertransport case requires that Ca^{2+} transport will deplete the vesicle lumen of K^+ and will give (at least transiently) different energetics and internal osmolarity than would be the case in the anion cotransport mechanism. The present communication addresses the problem of the relative contributions of cotransport and countertransport mechanisms and determines the most probable value of n (Fig. 1).

Figure 2 highlights an additional problem of the

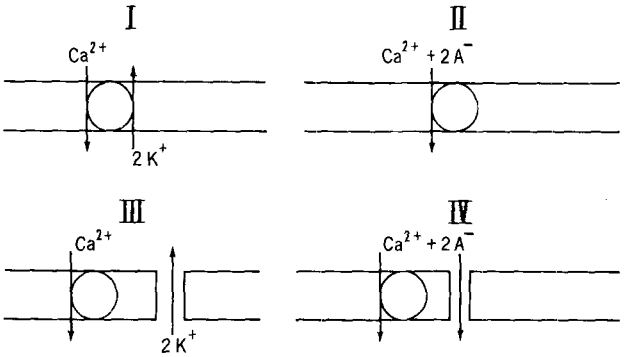

Fig. 2. Electrically silent *vs.* electrogenic model of action of the Ca-ATPase. Model *I* (cation countertransport) and Model *II* (anion cotransport) are electrically silent. Models *III* and *IV* involve electrogenic action of the Ca-ATPase coupled to electrically active permeabilities intrinsic to the SR

ATPase mechanism—whether the ATPase molecule is of itself sufficient to give rise to net transport (models *I* and *II*) or whether it functions in parallel with other (passive) permeability systems (models *III* and *IV*). We believe that the only valid criteria for choosing between models *I* or *III* or between models *II* or *IV* are (i) the demonstration that inhibition of the pump inhibits the anion or cation passive permeability, (ii) biochemical and functional isolation of the pump from the presumptive ion channels, and (iii) the demonstration of true electogenic function of the pump or of electrically active permeabilities capable of giving rise to membrane potentials in SR. Criteria (i) and (ii) have not been applied to the problem. There have been a number of reports of the generation of measurement of membrane potentials in isolated SR. McKinley and Meissner (1977, 1978) reported the existence of a monovalent cation channel in 2/3 of the SR vesicles. They reported the generation and measurement of cation gradient-dependent membrane potentials in the absence of permeable anions. A report has been made of the measurement of inside-positive membrane potential accompanying active transport (Åkerman & Wolff, 1979). The report was based on the behavior of an oxalol dye. Du Pont (1979) reported changes in thiodicarbocyanine dye fluorescence accompanying Ca^{2+} transport and interpreted them as the result of electrogenic pump function. More extensive work (Beeler & Martonosi, 1979; Russell, Beeler & Martonosi, 1979*a*, 1979*b*; Beeler, Russell & Martonosi, 1979) has shown that both the oxalol and oxycyanine dye binding equilibria respond to the internal divalent cation concentrations as well as to membrane potential. The conclusion of the aforementioned studies was that membrane potential changes do not occur in the SR during active transport. However, this cannot be taken as proof that

the ATPase does not function electrogenically since the intrinsic (electrically active) cation and anion permeabilities of the SR would be sufficient to mask the electrogenicity of the pump. Electrogenic behavior has been reported for the reconstituted Ca-ATPase by Zimniak and Racker (1978), but the effects were small, the basis of the measurements was ill defined, and the arguments were tenuous. In particular, they assumed that ANS^- measures membrane potential and that its signal can be calibrated using a valinomycin plus K^+ gradient "voltage clamp", a procedure against which we have specifically cautioned (Haynes & Simkowitz, 1977). Their report is discussed in greater detail at the end of this paper. It would seem that the present literature does not resolve the problem of whether the Ca-ATPase operated electrogenically or as a Ca^{2+}/Mg^{2+} or $Ca^{2+}/2K^+$ exchanger.

The goal of the present study is to test the mechanism of Fig. 1 in appropriately designed rapid kinetic experiments using a spectroscopic indicator of free Ca^{2+} in the SR lumen. In these studies we have used the ATPase-rich fraction of the SR (Meissner, 1975). As prepared in our laboratory, this material shows one major band under sodium dodecylsulfate (SDS) gel electrophoresis, attributable to the ATPase.

Materials and Methods

The preparation of the ATPase-rich SR fraction and the description of the stopped-flow spectrometer are given in the previous papers (Chiu et al., 1980; Chiu & Haynes, 1980). Active Ca^{2+} uptake experiments were performed at 23 °C in a medium containing either (*a*) buffer (0.6 M sucrose, 10 mM histidine, pH 7.2), 0.1 mM $MgCl_2$, 4×10^{-4} M EGTA, 4×10^{-4} M $CaCl_2$, 3×10^{-5} M ANS^-, 6 μM valinomycin, and 0.1 mg/ml SR protein or (*b*) buffer (0.6 M sucrose, 30 mM Tris-HCl, pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 4×10^{-4} M EGTA, 4×10^{-4} M $CaCl_2$, 3×10^{-5} M ANS^-, and 0.1 mg/ml SR protein. In both cases 5 μM valinomycin was added to the SR vesicles unless otherwise mentioned in the figure legend. Active uptake experiments were initiated by rapid mixing of ATP (in medium *a* or *b*) to a final concentration of 0.2 or 5 mM for *a* and *b*, respectively. The control mixing experiments in which no ATP was added, gave a flat response in type *a* experiment. However, a small fluorescence increase was observed in type *b* experimental condition in the absence of ATP. This is due to the effect of ANS^- binding to the SR to lower the free ANS^- in the SR incubation medium to about 90% of the concentration in the ATP medium. This small artifact was eliminated by adding a 10% excess of ANS^- to the reservoir containing the SR vesicles to equalize the free concentrations.

The quality of SR vesicles was tested for each isolation using the incubation medium of *a*. The $t_{\frac{1}{2}}$ of the preparations varies from 1 to 7 sec. Only those preparations with $t_{\frac{1}{2}}$ values ranging from 1 to 3 sec were used. A portion of the SR was studied on the day of isolation, and the remainder was quickly frozen and stored at −70 °C until used. The freezing and thawing had no discernible effect on the transport properties measured here. The method of estimation of the lumenal space is described in the previous paper (Chiu & Haynes, 1980).

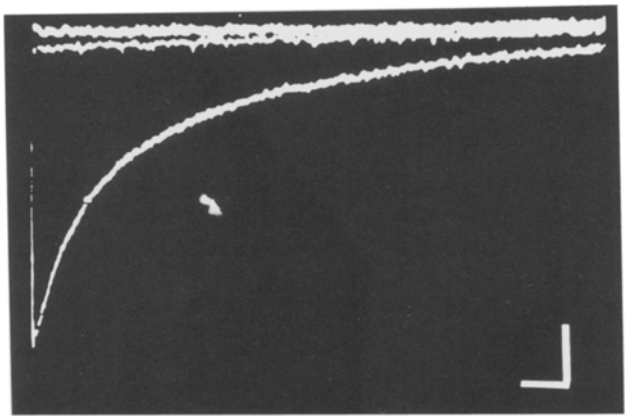

Fig. 3. ANS^- fluorescence increase induced by active transport of Ca^{2+}. The vertical axis is fluorescence in units of 0.2 volt/division; the horizontal axis is time in units of 5 sec/division. The downstroke is an artifact due to our choice of instrumental time constant (0.5 sec). The mixing configuration was 0.2 mg/SR protein, 3×10^{-5} M valinomycin *vs.* 2×10^{-4} M $MgCl_2$, 4×10^{-4} M ATP. Both reservoirs contained 3×10^{-5} M ANS^-, 0.6 M sucrose, 5 mM KCl, 30 mM histidine, pH 7.2, 4×10^{-4} M $CaCl_2$ and 4×10^{-4} M EGTA. The experiment was carried out at 23 °C

Proof that ANS^- Responds to Surface Potential Changes and not to Conformational Changes in the Ca-ATPase

The first paper in this series (Chiu et al., 1980) shows that the ANS^- fluorescence signal arises primarily from ANS^- bound to the lipid bilayer regions. Several types of experiments were done to show that the fluorescence increase observed upon active transport is due solely to the active Ca^{2+} accumulation and the concomitant increase in $\exp(e\psi_{o,i}/kT)$ as described in the previous paper (Chiu & Haynes, 1980). (*a*) Addition of Mg-ATP in a Ca^{2+} free medium to the SR does not affect the fluorescent signal of membrane bound ANS^-. (*b*) In the presence of low concentrations of $HgCl_2$ (5×10^{-5} M), no fluorescence increase is observed when ATPase activity is initiated by the addition of ATP. This Hg^{2+} concentration is capable of releasing actively preloaded Ca^{2+} without significantly inhibiting the ATPase function of the enzyme (Shamoo & MacLennan, 1975; V.C.K. Chiu and D.H. Haynes, *in preparation*). (*c*) No fluorescence increase is observed upon addition of ATP to aged SR. However, under such conditions, the ATPase pump is still turning over as indicated by the ATP hydrolysis rate. (*d*) ATP-induced fluorescence can be totally reversed to zero when the Ca^{2+} ionophore A23187 (5×10^{-7} M) is added. Experiments *b*, *c*, and *d* eliminate the possibility of conformational change of the ATPase or ANS^- being buried deeper in the membrane as the explanation for the observed fluorescence increase during active Ca^{2+} uptake. (*e*) Ca^{2+} active uptake experiment in which the protein is excited at 290 nm and the ANS^- emission is monitored gives the same $t_{\frac{1}{2}}$ value as compared to direct ANS^- excitation. This result indicates ANS^- molecules in the neighborhood of the proteins behave very similar to ANS^- molecules in the lipid region.

Results

1) Internal Free Ca^{2+} Concentration under Active Uptake Conditions

Figure 3 shows a stopped-flow active uptake experiment in which 0.2 mg/ml of ATPase-rich SR pre-

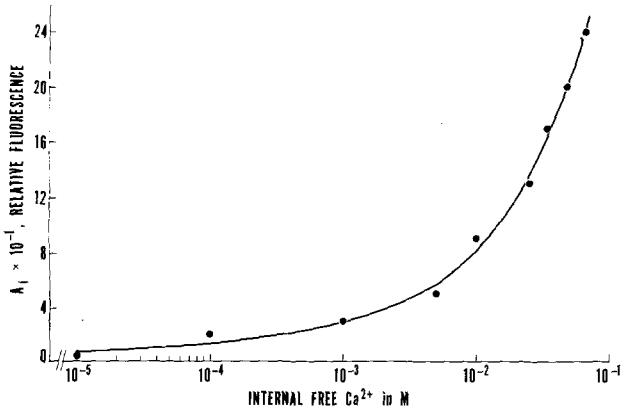

Fig. 4. Amplitude of ANS^- fluorescence arising from the inside surface as a function of the internal Ca^{2+} concentration. The data was obtained from Ca^{2+} jump experiments in the absence of ATP. The total amplitude of the slow phases ($t_{\frac{1}{2}} > 3$ msec) is plotted against the Ca^{2+} concentration after mixing. The mixing configuration was as follows: Reservoir *A*: 0.2 mg/ml SR and 3×10^{-5} M valinomycin. Reservoir *B*: variable concentrations of Ca^{2+}. Both reservoirs contained 0.6 M sucrose, 1×10^{-4} M $MgCl_2$, 5 mM KCl, 4×10^{-4} M EGTA and 30 mM histidine, pH 7.2. Identical results were obtained in the presence of catalytic concentrations of X537A, indicating that complete equilibration across the membrane had been achieved

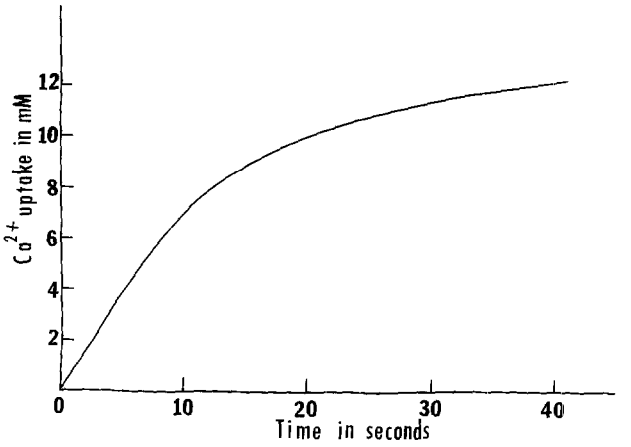

Fig. 5. Progress curve for active Ca^{2+} uptake. The fluorescence progress curve of Fig. 3 was transformed by the fluorescence *vs.* $[Ca^{2+}]_i$ relationship given in Fig. 4

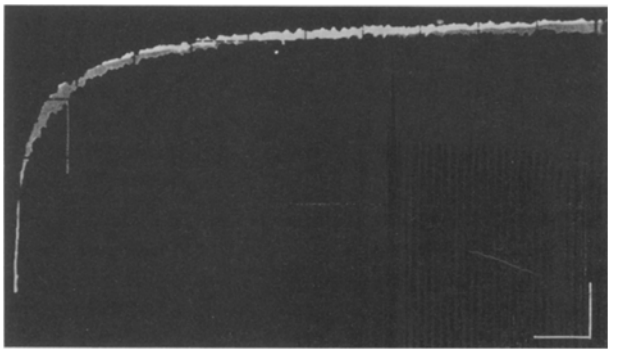

Fig. 6. Stopped-flow ANS^- fluorescence increase induced by active Ca^{2+} transport. The vertical axis is fluorescence increase in units of 0.2 volt/division; the horizontal axis is time after mixing in units of 5 sec/division. The mixing configuration was 0.2 mg/ml of SR protein, 6×10^{-6} M valinomycin *vs.* 5 mM ATP. Both reservoirs contain 30 mM tris-HCl, pH 7.0, 50 mM KCl, 4×10^{-4} M EGTA, 4×10^{-4} $CaCl_2$, and 3×10^{-5} M ANS^-. The experiment was carried out at 23 °C. The half-time for ANS^- permeation was 10 msec under these conditions. The same results were observed one-half or 2 times the valinomycin concentration

equilibrated with valinomycin and ANS^- is rapidly mixed with ATP and Mg^{2+}. The medium contained K^+ and Ca^{2+}, buffered to a low concentration (ca. 2.5×10^{-5}) by EGTA. A single process of fluorescence increase is seen with a $t_{\frac{1}{2}}$ of 6 sec. The fluorescence increase is due to the active accumulation of Ca^{2+} in the intravesicular space, resulting in an increase in $\exp(e\psi_{o,i}/kT)$ as described in the previous papers (Chiu et al., 1980; Chiu & Haynes, 1980). Repetition of the experiment omitting the ATP demonstrated that the fluorescence was due to active transport.

The fluorescence *vs.* $[Ca^{2+}]_i$ relationship was determined by performing Ca^{2+}-jump experiments under the conditions of Fig. 3 but in the absence of ATP. Figure 4 is a calibration curve constructed from the passive transport data. The amplitude of the phases corresponding to the transmembrane step ($t_{\frac{1}{2}} > 3$ msec) is plotted against the internal Ca^{2+} concentration. The previous paper (Chiu & Haynes, 1980) shows that the internal and external Ca^{2+} concentrations become equal at the end of the slow phase.

The progress curve for the fluorescence increase induced by active transport (Fig. 3) was transformed by the use of the calibration curve (Fig. 4) to yield a Ca^{2+} uptake curve under the assumption that $[K^+]_i = [K^+]_o$ during the reaction. This condition obtains for the 2/3 of the vesicles which are KCl permeable with a 2-sec half-time (Chiu & Haynes, 1980). Figure 5 is the result of the transformation showing the free internal Ca^{2+} concentration as a function of time after initiation of active uptake. The figure shows that the free internal Ca^{2+} concentration reaches 12 mM with a half-time of approximately 9 sec.

A long $t_{\frac{1}{2}}$ was observed because the low Mg^{2+} and K^+ concentrations used were insufficient for maximal activation of the ATPase pump. When these ions are present at more optimal concentrations, much faster rates are observed. The fluorescent amplitudes are reduced since the high concentrations of these ions bring the initial values of $\exp(e\psi_{o,i}/kT)$ closer to unity (*cf.* Fig. 7, Chiu et al., 1980). Figure 6

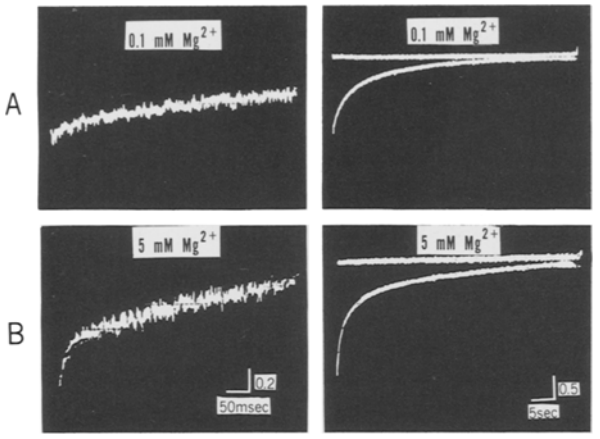

Fig. 7. Effects of low and high Mg^{2+} on the progress curve for Ca^{2+} uptake. The reaction conditions are essentially the same as in Fig. 3 except that Mg^{2+} was present in both reservoirs. The KCl concentration was 5 mM (both reservoirs) and the Mg^{2+} concentrations are indicated in the figure. The vertical and horizontal sensitivities for the traces on the left-hand portion of the figure are 0.2 volt/division and 50 msec/division, respectively. The sensitivities for the right-hand portion of the figure are 0.5 volt/division and 5 sec/division, respectively. Repetition of the experiments without ATP gave a flat response. Repetition of the experiments with aged leaky SR vesicles or normal SR vesicles treated with low concentrations of Hg^{2+} also gave a flat response

Table 1. Effect of increasing Mg^{2+} concentration on the ATP induced fluorescence enhancement in 5 mM KCl

$[Mg^{2+}]$ in M	ATP-induced amplitude and $[Ca^{2+}]_i$ achieved in 50 msec		Total ATP-induced fluorescence amplitude	Maximal steady-state $[Ca]_i$ (mM)	Fluorescent $1/t_{\frac{1}{2}}$ overall (sec^{-1})
	Amplitude	$[Ca^{2+}]_i$ (mM)			
1×10^{-4}	0.44	0.3 ± 0.1	1.7	10	0.33
2×10^{-4}	0.65	–	2.0	–	0.50
5×10^{-4}	0.75	–	2.0	–	1.00
1×10^{-3}	0.80	–	2.2	–	1.00
3×10^{-3}	0.84	–	2.2	–	1.00
5×10^{-3}	0.92	2.0 ± 0.5	2.2	35 ± 5	1.00
10×10^{-3}	0.70	2.0 ± 0.5	2.2	40 ± 5	0.50

Ca^{2+} active uptake conditions are given in the legend of Fig. 3, except that Mg^{2+} is present in both of the mixing reservoirs and the valinomycin concentration is 6×10^{-6} M. The ATP concentration was held constant at 2×10^{-4} M (after mixing).

shows the results of an experiment in which SR is preincubated with 50 mM KCl and 5 mM $MgCl_2$ and active transport initiated by adding 5 mM ATP. The $t_{\frac{1}{2}}$ of the fluorescent rise associated with the active uptake process is about 600 msec under these reaction conditions. Conversion of the ATP-induced fluorescence amplitude to $[Ca^{2+}]_i$ by determination of a A_i *vs.* $[Ca^{2+}]_i$ calibration curve for the reaction medium indicates that the internal free Ca^{2+} concentration reaches 70 ± 20 mM.

The experiment of Fig. 6 was repeated in a fluorometer, and EGTA was added to a final concentration of 10 mM after the maximal fluorescence amplitude was achieved. This resulted in the slow decrease in fluorescence amplitude due to pump reversal cited in the previous paper (Chiu & Haynes, 1980). The $t_{\frac{1}{2}}$ for the reversal was ca. 100 sec.

2) Effect of Mg^{2+} on the Active Transport Reaction

The effect of Mg^{2+} (Ebashi & Lipmann, 1962) on the active uptake progress curve was studied. We observed that increasing the Mg^{2+} concentration from 10^{-4} M increases both the rate of the active transport reaction and the maximal steady-state internal Ca^{2+} concentration achieved. Oscilloscope traces of the progress of the reaction are shown in Fig. 7. The figure shows recordings on the fast and the slow time scales for the reactions catalyzed by two different Mg^{2+} concentrations (0.1 and 5 mM). Quantitation of the rate and amplitudes is given in Table 1. Four mechanistic features of the Mg^{2+} catalysis were observed: (i) Increasing the Mg^{2+} concentration results in an increase in the total fluorescence amplitude. Using the techniques of quantification described above, the maximal steady-state Ca^{2+} level increased from about 10 mM for 10^{-4} M Mg^{2+} to 40 ± 5 mM for 10 mM Mg^{2+}. (ii) At high Mg^{2+} concentrations (5–10 mM) the progress curve becomes distinctively biphasic, with an initial burst of fluorescence increase observed in the first 30–40 msec. An internal Ca^{2+} concentration of 3 mM is achieved in this time. (iii) Increasing the Mg^{2+} concentration increased the rate of fluorescence increase averaged over the first 500 msec. This effect was also observed at concentrations where the rapid phase of increase could not be discerned. (iv) Increasing the Mg^{2+} concentration also increased the overall rate of the reaction as measured by the $1/t_{\frac{1}{2}}$ value. The effects of increasing the Mg^{2+} concentration up to 3 mM are consistent with the view that increasing levels of Mg-ATP and Mg^{2+} cause higher rates of phosphorylation and dephosphorylation, respectively (Kanazawa et al., 1971; Froehlich & Taylor, 1975, 1976). Increasing the rates of these partial reactions should speed up the overall rate of pump turnover. In section 4, the catalytic effect of Mg^{2+} will be localized to the inner side of the membrane.

Table 2. The effect of increasing K^+ concentration on active uptake at high Mg^{2+} and ATP concentrations

First phase			Second phase		Overall reaction			Average rate
K^+ (mM)	$A_{i,1}$	$1/t_{\frac{1}{2}(1)}$ (sec^{-1})	$A_{i,2}$	$1/t_{\frac{1}{2}(2)}$ (sec^{-1})	A_i	$1/t_{\frac{1}{2}}$ (sec^{-1})	$(Ca^{2+})_i$ (mM)	(mM sec^{-1})
0.1	0.36	10	0.54	0.25	0.90	0.5	17±2	3.6±0.4
1.0	0.40	13	0.50	0.40	0.90	1.25	17±2	8.5±1.0
10	0.60	13	0.50	0.50	1.10	2.00	35±2	28±2
25	[a]	[a]	[a]	[a]	1.20	2.00	40±2	32±2
50	[a]	[a]	[a]	[a]	1.2	1.66	40±3	26±2
100	[a]	[a]	[a]	[a]	1.2	1.66	64±5	42±2

The active uptake conditions are the same as in Fig. 6, except that the KCl concentration was varied and the final Mg^{2+} and ATP concentrations were 7.5 and 5.0 mM, respectively. Identical results were observed with one-half or twice the valinomycin concentration. The internal Ca^{2+} concentration was determined by the passive calibration procedure outlined in the text. The average rate is a rough estimate of the rate calculated as 0.4 $(Ca^{2+})_i$ $(1/t_{\frac{1}{2}})$. (*cf.* Tables 3 & 4, Chiu & Haynes, 1980).

[a] Fast and slow phases could not be resolved.

3) Effect of Monovalent Cations on Active Ca^{2+} Transport

Monovalent cations have been shown to stimulate Ca-ATPase and Ca^{2+} transport activities under appropriate conditions for both skeletal and cardiac sarcoplasmic reticulum. K^+ is the most effective monovalent cation (Duggan, 1977; Shigekawa & Dougherty, 1978*a*, 1978*b*). The effect correlates with an increase in ATPase activity. Duggan (1977) has shown that the monovalent cation stimulation effect is due to its effect to speed up the rate. The Ca/ATP stoichiometry is not affected. The evidence for tight coupling of Ca^{2+} transported and ATP split rules out a passive leak as a determinant of the level of maximal steady-state uptake. The effect of monovalent cations on the pump rate has been attributed to their ability to increase the rate of dephosphorylation of the enzyme (Duggan, 1977; Shigekawa & Dougherty, 1978*a*, 1978*b*). It is noted that this effect can be explained by the model of Fig. 1 either by catalysis of step 7 or by occupation of the carrier to allow it to return the ionophore to the outside surface (step 9), or by both mechanisms.[1] Table 2 shows the effect of the K^+ concentration on the rate of Ca^{2+} uptake and on the maximal steady-state concentrations achieved. These experiments were performed with 7.5 mM Mg^{2+} and 5 mM ATP, under which condition the pump is fully activated. As is shown in the table, two phases of fluorescence increase are observed. Increasing the K^+ concentrations increases the rate of both, with a larger effect seen for the second phase. Above 10 mM K^+, the rate of the second phase is increased such that the fast reaction of the first phase is no longer resolved as a separate phase. The maximal effect of K^+ on the rate of the slow phase is a 13-fold stimulation. The stimulatory effect on the steady-state Ca^{2+} concentration is much smaller. Thus the presence of K^+ affects the pumping rate but has little effect on the ability of the pump to maintain a Ca^{2+} gradient. This suggests that the gradient is determined by the ability of the pump to compete with a "passive leak" but rather by the tightness of coupling between the phosphorylation-dephosphorylation steps and the pump's translocation reactions of the Ca^{2+} binding site. This is exactly what would be expected from our conclusions (Chiu & Haynes, 1977; Haynes & Chiu, 1977, 1978) that the passive Ca^{2+} permeability of SR measured in the absence of ATP is due to partial reactions of the pump (Chiu & Haynes, 1980). The relative abundance of the conformational and occupational states will not be the same in the ATP-energized case as in the passive case, and the maximal steady-state uptake cannot be simply modelled as an active transport process and a passive "leak" operating independently of each other. In the Discussion section we will argue that the stimulatory effect of high K^+ on the transport rate is due primarily to the role of the ATPase as a $2K^+$ for Ca^{2+} exchanger, as shown schematically in Model *I* of Fig. 2. High internal K^+ concentrations are necessary for maximal Ca^{2+} to be accumulated in a short time. The maximal steady-state uptake is less a function of the K^+ concentration since passive KCl leak processes operating independently of the pump allow the intravesicular K^+ to be replenished.[2]

The effect was also investigated at low Mg^{2+} concentrations under which condition the pump is less than maximally activated. Figure 8 shows the effect of variation of the K^+ concentration (5 *vs.* 50 mM) on the active uptake progress curve observed in the presence of 1×10^{-4} M Mg^{2+}. Increasing the K^+ concentration does not bring about an increase in the overall rate of maximal steady-state uptake. However, the increased K^+ concentration does give rise to a

[1] Inesi et al. (1978*a*, 1978*b*) have shown that internal Ca^{2+} binds to the active site with a K_d of about 1 mM. This internal concentration is achieved within a single pump turnover. K^+ binding to the carrier would decrease the Ca^{2+} binding. For an accelerative effect of K^+ it would be necessary for it to remain bound to the active site.

[2] Since this mechanism operates on a 2-sec time scale in the KCl permeable vesicles and much more slowly in the KCl impermeable vesicles, we did not assume $[K^+]_i = [K^+]_o$ in order to calculate $[Ca^{2+}]_i$ at the end of the first phase in the low $[K^+]$ cases.

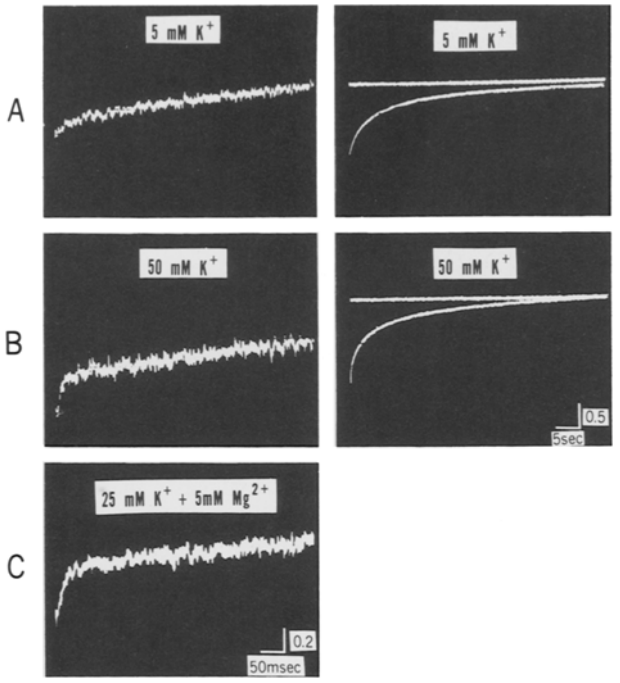

Fig. 8. Effect of the K^+ concentration on the active uptake reaction. The experimental conditions are the same as in Fig. 4 except that Mg^{2+} was present in both reservoirs at 10^{-4} M concentration, unless otherwise indicated. The K^+ concentrations are given in the figure. The vertical and horizontal scales for the traces on the left are 0.2 volt/division and 50 msec/division, respectively. The vertical and horizontal scales for the traces on the right are 0.5 volt/division and 5 sec/division, respectively

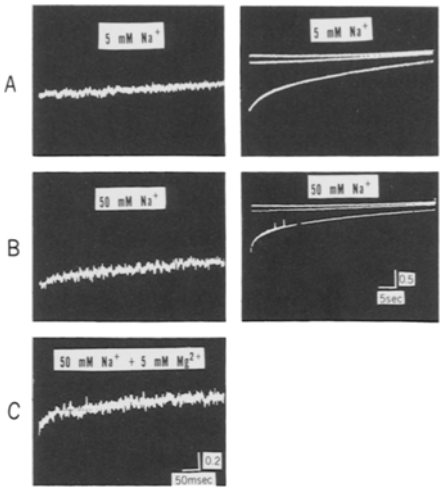

Fig. 9. Effect of Na^+ concentration on the active uptake reaction. Conditions are as in Fig. 8

Table 3. Effect of KCl concentration on active uptake at low Mg^{2+} (1×10^{-4} M) and ATP (2×10^{-4} M) concentrations

KCl (mM)	Amplitude of fast phase[a]	$\Delta[Ca^{2+}]_i$ for fast phase (mM)	Amplitude of slow phase	$\Delta[Ca^{2+}]_i$ slow phase (mM)	$1/t_{\frac{1}{2}}$ overall (sec^{-1})	$[Ca^{2+}]_i$ final (mM)
1	<0.05	[b]	1.7±0.1	[b]	0.2	[b]
5	<0.05	[b]	1.7±0.1	[b]	0.2	[b]
10	<0.05	[b]	1.8±0.1	[b]	0.2	[b]
50	0.3±0.05	[b]	1.4±0.05	[b]	0.2	[b]
100	0.4±0.05	2.0±0.05	1.2±0.05	12±1	0.20	14±1
200	0.6±0.05	[b]	1.0±0.1	[b]	0.5	[b]
400	1.0±0.05	8.0±0.05	0.6±0.05	12±1	4.0	20±1
25 mM K^+ + 5 mM Mg^{2+}	0.40±0.05	[b]	1.5±0.1	[b]	0.67	[b]

The experimental conditions are as given in Fig. 3 except that Mg^{2+} was present in both reservoirs and the concentration of valinomycin was 6×10^{-6} M.

[a] The fast phase was complete in 50 msec or less.
[b] Not determined.

rapid initial phase. The results for K^+ stimulation of low Mg^{2+} concentrations are shown in Table 3. The amplitude of the initial phase increases in amplitude with increasing K^+ concentrations. Figure 9 and Table 4 show that similar effects are observed with Na^+; thus under conditions where the pump is less than maximally activated, increasing the monovalent cation (M^+) concentration still results in an increase in the rate of uptake. In the discussion section, these effects will be interpreted in terms of the mechanisms of Fig. 1. The primary effect of M^+ is seen as the competition with Ca^{2+} for occupation of the inwardly-directed Ca^{2+} binding site and the countertranslocation of the bound cation.

The M^+ specificity of the enhancement effect was investigated at high Mg^{2+} concentrations with the pump fully activated. Table 5 shows that valinomycin is able to shuttle the M^+-ANS^- pairs across the membrane sufficiently rapidly that the kinetics of Ca^{2+}

Table 4. Effect of NaCl concentration on active uptake at low Mg^{2+} (10^{-4} M) and low ATP (2×10^{-4} M) concentrations

$[Na^+]$ (mM)	Amplitude of fast phase[a]	Amplitude of slow phase	$1/t_{\frac{1}{2}}$ overall (sec^{-1})
5	<0.05	1.7±0.1	0.08
25	<0.05	1.5±0.1	0.10
50	0.10±0.05	1.3±0.1	0.13
100	0.18±0.05	1.2±0.1	0.13
50 mM Na^+ + 5 mM Mg^{2+}	0.30±0.05	1.3±0.1	0.14

Experimental conditions are as in Table 3.

[a] The fast phase was complete in 50 msec or less.

transport are still faithfully resolved. Table 6 shows that there is ca. a twofold range of variation in efficacy of stimulation among the monovlent cations, with the order of effectiveness $K^+ > Cs^+ > Rb^+ > Na^+$. This can be compared with the cation specificity observed (Duggan, 1977) for Ca^{2+} uptake rate in an oxalate loading system ($K^+ > Na^+ > NH_4^+ = Rb^+ = Cs^+ > Li$), covering a threefold range of V_m and a fivefold range of K_m.

4) Sites for K^+ and Mg^{2+} Stimulation of Transport

It is possible to use the ANS^- method to study the functional location of K^+ and Mg^{2+} ions. This can be accomplished by measuring the Ca^{2+} transport rate observed when the ion is supplied at the instant when ATP is added, and by comparing this rate with that observed when the SR is preincubated with the ion. In the first case the ions are available to the stimulatory site at the instant when the reaction is started. In the second case ions will not be directly available to the site if the site is located on the inside surface behind a permeability barrier. Table 7 summarizes our findings on active Ca^{2+} uptake under the same ionic conditions but with different stopped-flow mixing configurations. The table also contains a number of control experiments carried out under passive conditions. The fluorescent amplitudes and $t_{\frac{1}{2}}$'s for their attainment are given. Experiments 1, 3, 5 and 8 repeat our previous experiments showing the KCl and Mg^{2+} are necessary for maximal rates of active transport. The requirement for K^+ and Mg^{2+} in the SR lumen is demonstrated in experiments in which these ions are omitted from the preincubation medium and are added simultaneously with the ATP. If the cations exert their effects at the inside surface, the active transport rates will be retarded, due to the requirement of passive transport of the K^+ and Mg^{2+} across the membrane. The previous

Table 5. Effectiveness of valinomycin-cation combinations in transporting ANS^- across the SR membrane

Monovalent cations	$t_{\frac{1}{2}}$ (msec)	Relative rate in $1/t_{\frac{1}{2}}$ (sec^{-1})
Na^+	70	14
K^+	20	50
Rb^+	20	50
Cs^+	60	17

The experiments were carried out with the following mixing configuration (0.2 mg/ml SR protein, 6×10^{-6} M valinomycin) *vs.* (6×10^{-5} M ANS^-). Both reservoirs contained 30 mM Tris-HCl at pH 7.0, 50 mM monovalent cations, 5 mM, $MgCl_2$, 4×10^{-4} M EGTA dissolved in Tris-base, 4×10^{-4} M $CaCl_2$. The temperature was 23 °C.

Table 6. Stimulation of ATP-induced fluorescence enhancement by different monovalent cations with chloride as an anion

Monovalent cations in 100 mM	$1/t_{\frac{1}{2}}$ (sec^{-1}) of overall reaction
Na^+	0.67 ± 0.1
K^+	1.45 ± 0.2
Rb^+	0.67 ± 0.1
Cs^+	1.00 ± 0.1

Ca^{2+} active uptake conditions are given in the legend of Fig. 6 with the appropriate choice of monovalent cation at 100 mM concentration. When the ionophore concentration was doubled, the active Ca^{2+} transport rate remains unchanged.

Table 7. Functional location of the stimulatory effect of K^+ and Mg^{2+} on the rate of Ca^{2+} transport

Expt #	Active/passive	K^+ perturbation $[K^+]_{preincub.} \rightarrow [K^+]_{final}$ (mM)	Mg^{2+} perturbation $[Mg^{2+}]_{preincub.} \rightarrow [Mg^{2+}]_{final}$ (M)	A_1	$t_{\frac{1}{2},1}$	A_2	$t_{\frac{1}{2},2}$ (sec)
1	Active	5 → 5	$1 \times 10^{-4} \rightarrow 1 \times 10^{-4}$	–	–	+2.3	6
2	Active	0 → 5	$1 \times 10^{-4} \rightarrow 1 \times 10^{-4}$	<0.3	–	+2.2	15
3	Active	0 → 0	$1 \times 10^{-4} \rightarrow 1 \times 10^{-4}$	–	–	+3.0	27
4	Passive	0 → 5	$1 \times 10^{-4} \rightarrow 1 \times 10^{-4}$	−0.3	2 sec	−0.2	65
5	Active	5 → 5	0 → 1×10^{-4}	–	–	+2.5	13
6	Active	5 → 5	0 → 0	–	–	+2.2	25
7	Passive	5 → 5	$1 \times 10^{-4} \rightarrow 1 \times 10^{-4}$	–	–	+0.5	150
8	Active	100 → 100	$1 \times 10^{-4} \rightarrow 1 \times 10^{-4}$	+0.2	100 msec	+1.7	10
9	Active	0 → 100	$1 \times 10^{-4} \rightarrow 1 \times 10^{-4}$	+0.8	100 msec	+0.9	25
10	Passive	0 → 100	$1 \times 10^{-4} \rightarrow 1 \times 10^{-4}$	−0.4	200 msec	−0.6	45

All experiments were carried out using the same basic medium consisting of 0.6 M sucrose, 10 mM histidine pH 7.2, 4×10^{-4} M Ca-EGTA and 3×10^{-5} M ANS^-. The SR was preincubated with 6 μM valinomycin. Active uptake ("active") was initiated by the addition of ATP to a final concentration of 2×10^{-4} M. The concentrations of K^+ and Mg^{2+} in the preincubation and their final concentrations after mixing are as given. "Passive" denotes the control experiments in which the ionic preincubations were made in the absence of ATP.

paper (Chiu & Haynes, 1980) and Table 7 show that the approximate half-time for the passive equilibration are ca. 2 sec and ca. 100 sec for KCl, and ca. 100 sec for Mg^{2+}. These half-times are long enough for significant retardation to be seen in the active transport reaction. The comparison of Expts. 1 *vs.* 2 and 8 *vs.* 9 shows that the a greater than twofold decrease in the active transport rate is brought about by ommission of K^+ from the preincubation medium. The control experiment (Expt. 4) for the low $[K^+]$ condition shows that the fluorescent amplitude attributable to the $[K^+]$ manipulation is less than 10% (opposite sign) of the amplitude of the active uptake reaction. For the high $[K^+]$ condition, the amplitude (slow phase) attributable to the $[K^+]$ manipulation is about 30% (opposite sign) of that of the active uptake reaction (Expts. 8 and 10). The slow phase of the passive $[K^+]$ equilibration is slower than the $t_{\frac{1}{2}}$ for active uptake reaction. It is clear that lack of K^+ pre-equilibration has produced a significant decrease in the overall rate of the active transport reaction. This is most readily explained as a requirement of K^+ in the SR lumen, and the result is in agreement with the predictions of the model of Fig. 1. The requirement for added K^+ is not absolute since counter-transport can also be facilitated by adventitious monovalent cations in the medium.

Experiments 1, 5 and 6 show that omission of Mg^{2+} from the preincubation medium has a similar effect on the transport rate. The passive control experiment (7) shows only 20% of the amplitude of the active uptakes experiment and has a much longer time course. This can also be taken as evidence that Mg^{2+} facilitates the active transport reaction at a site accessible to the inside surface. In the discussion section we will show that this effect, observed at Mg^{2+} concentrations which are small compared with those of the transported Ca^{2+}, is probably related to the effect of internal Mg^{2+} on the dephosphorylation step (7, Fig. 1). The slow rate observed by the complete omission of Mg^{2+} from the medium is also a consequence of the fact that Mg-ATP is the true substrate for the ATPase (Vianna, 1975). We attribute the finite rate observed in the absence of added Mg^{2+} to a small amount of Mg^{2+} carried over in the SR preparation or in the ATP.

5) Effect of Ca^{2+} Concentration on the Ca^{2+} Transport Rate and Steady-State Internal Concentration

When the free Ca^{2+} concentration is increased from 29 μM to 10^{-4} M in the active uptake medium containing suboptimal Mg^{2+} concentrations, the initial Ca^{2+} transport rate, the overall reaction rate ($1/t_{\frac{1}{2}}$), and the total Ca^{2+} accumulation are decreased by a factor of two (Table 8). In the Discussion section we will show that this is due to the inhibitory effect of Ca-ATP on the rate of Ca^{2+} transport and the steady-state Ca^{2+} accumulation.

Table 8. Effect of Ca^{2+} on the ATP-induced fluorescence increase and $1/t_{\frac{1}{2}}$ of the overall Ca^{2+} transport rate

$[Ca^{2+}]_{free}$	Initial ATP-induced fluorescence amplitude in 250 msec	Total ATP-induced fluorescence amplitude due to Ca^{2+} uptake	Fluorescent $1/t_{\frac{1}{2}}$ (sec^{-1}) of overall reaction
25 μM	0.6	2.3	0.33
10^{-4} M	0.3	1.4	0.17

Ca^{2+} active uptake conditions are given in the legend of Fig. 3, except that Mg^{2+} is present in both of the mixing reservoirs and the concentration of valinomycin is 6×10^{-6} M. The concentrations of free Ca^{2+} are given in the table. In the high Ca^{2+} (10^{-4} M) uptake experiment, small additional ATP was added (8×10^{-5} M) in order to maintain the same Mg-ATP level as in the low Ca^{2+} concentration experiment. The fluorescence levels before the initiation of transport were nearly identical. The ATP-induced amplitudes are thus a valid measure of the internal Ca^{2+} concentration.

6) Requirement of Prior Occupation of the High Affinity Ca^{2+} Binding Site of the Carrier

In a series of experiments presented in Fig. 10 and summarized in Table 9, we explored the effect of order of addition of Ca^{2+} and ATP on the active transport reaction. According to the scheme of Fig. 1 under the assumptions of fast kinetics of steps 1–4, the order of addition should be immaterial. Table 9 gives two experiments which show that this expectation is not fulfilled. In one experiment, SR vesicles were suspended in a Ca^{2+}-free medium and the reaction was initiated by adding ATP and buffered Ca^{2+} ($[Ca] = 29$ μM). The other experiment was done according to our normal protocol with the SR preequilibrated with the buffered Ca^{2+} and the reaction initiated by adding ATP. The compositions of the media of the two experiments are identical after the 3-msec mixing time of the instrument.

Figure 10 shows that the Ca^{2+} jump experiment differs from the control experiment in three respects. Firstly, the initial Ca^{2+} transport rate is slower than the control value. Secondly, the total net Ca^{2+} accumulation is lower than that of the control. Thirdly, the vesicles lacking Ca^{2+} pre-equilibration show a delayed release phenomenon indicated in the second and third sweeps in Fig. 10. Observation *1* would not be expected if the rates of steps 1–4 were all

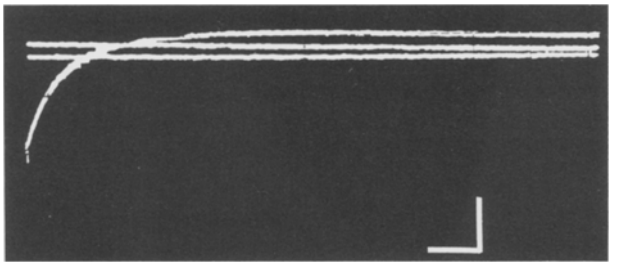

Fig. 10. Requirement of prior occupation of the pump with Ca^{2+}. The stopped-flow ANS^- fluorescence increase induced by active transport of Ca^{2+} is shown. The vertical and horizontal axes are 0.2 volt/division and 5 sec/division, respectively. The SR vesicles had been preincubated with 4×10^{-4} M EGTA for 10 min. The mixing configuration was 0.2 mg/SR protein, 6×10^{-6} M valinomycin, 4×10^{-4} M EGTA *vs.* 4.8×10^{-4} M ATP, 4×10^{-4} M EGTA, 8×10^{-4} M $CaCl_2$. Both reservoirs contained 3×10^{-5} M ANS^-, 0.6 M sucrose, 2×10^{-4} MgCl, 5 mM KCl, 30 mM histidine, pH 7.2. The experiment was carried out at 23 °C

rapid and were roughly equivalent. A Ca^{2+} deprivation effect on the kinetics of $E \sim P$ formation has been reported by Froehlich (1978). This suggests that the kinetics of the first turnover were altered. Observations *2* and *3* cannot be accounted for by the kinetic scheme of Fig. 1. A retardation of the initial rate could be explained, but after the first pump turnover all subsequent turnovers should be a function of only the instantaneous substrate and ion concentrations and should not depend on the history of the enzyme. This leads us to the conclusion that prior occupation of the high affinity Ca^{2+} binding site of the pump is necessary for the conformational or aggregational stability of the transport system. Ca^{2+} deprivation would favor conformational or aggregational forms having characteristics consistent with observations *1–3* and the rates of transformation between the two forms are slow, having $t_{\frac{1}{2}}$ values greater than 2 sec (*cf.* Fig. 10). The change is reversible since we observed that if Ca^{2+} was added back to the SR 45 sec before initiation of the reaction, the progress curve for uptake is identical to the control curve. Thus the change is reversible, with a $t_{\frac{1}{2}}$ between 2 and 45 sec. When the period of Ca^{2+} deprivation is extended to times longer than 2 hr, irreversible changes occur as evidenced by the inability of a 6-hr incubation with Ca^{2+} to reverse the change in the uptake curve. The kinetics of irreversible inactivation of the pump have been reported by McIntosh and Berman (1978).

7) The Effect of Valinomycin on Ca^{2+} Uptake Kinetics and Steady-State Levels

In the present studies we have used valinomycin as a means for translocating ANS^- across the membrane

Table 9. Effect of prior exposure to Ca^{2+} on Ca^{2+} uptake

	Initial ATP induced fluorescence amplitude in 250 msec	Total ATP induced fluorescence amplitude	Fluorescent $1/t_{\frac{1}{2}}$ (sec^{-1}) of overall reaction
1 Active Ca^{2+} uptake with Ca^{2+} preincubation (prior exposure)	0.30	1.8	0.28
2 Active Ca^{2+} uptake without Ca^{2+} preincubation	0.20	1.1	0.50
3 Active Ca^{2+} uptake without Ca^{2+} preincubation, but Ca^{2+} was added before the experiment was performed such that the mixing configuration was the same as experiment 1.	0.30	1.8	0.28

In experiment 1, the Ca^{2+} uptake conditions are given in the legend of Fig. 3, except that Mg^{2+} is present in both of the mixing reservoirs and the concentration of valinomycin is 6×10^{-6} M. In experiment 2, the active uptake conditions are the same as experiment 1 except that the SR has been incubated for 10 min with 4×10^{-4} M EGTA. The Ca^{2+} concentration in the other reservoir is doubled, such that the reaction conditions are identical to experiment 1, as soon as the reactions are mixed by the stopped flow apparatus. The conditions in experiment 3 are essentially the same as experiment 2 except that Ca^{2+} is added to the incubation medium after the 10-min EGTA incubation. The time between Ca^{2+} addition and the initiation of active uptake is 5 min.

such that it is competent to register the kinetics of the uptake process. However, in the previous study (Chiu & Haynes, 1980) we observed that valinomycin is capable of accelerating the passive movement of KCl across the SR membrane. It would seem that this could have an influence on the progress curve for active transport under conditions where internal K^+ was depleted by a $Ca^{2+}/2K^+$ countertransport mechanism. Alternatively, Zimniak and Racker (1978) have proposed an electrogenic pump function of the reconstituted ATPase and explained a small effect of valinomycin on the transport on the basis of its ability to move charge across the membrane. We have carried out experiments to determine whether valinomycin affects the active transport process.

Figure 11 shows active Ca^{2+} uptake by the SR vesicles in the absence (*A*) and in the presence (*B*) of 6×10^{-6} M valinomycin. The reaction medium contains 50 mM KCl, 0.1 mM Mg^{2+}, 0.4 mM Ca^{2+}, 0.4 mM EGTA, 10 mM histidine, pH 7.2, 600 mM sucrose, 0.03 mM ANS^- and 0.1 mg/ml SR protein. The reaction was initiated by adding 2×10^{-4} M ATP. The

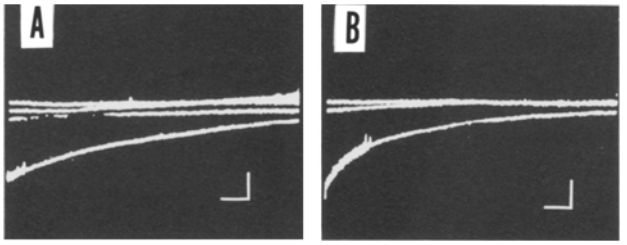

Fig. 11. Stopped-flow ANS^- fluorescence increase induced by active transport of Ca^{2+} in absence (*A*) and presence (*B*) of 6×10^{-6} M valinomycin. The vertical and horizontal axes are 0.5 volt/division and 5 sec/division, respectively. The mixing configuration was 0.2 mg/ml of SR protein (± valinomycin) *vs.* 2×10^{-4} M ATP. Both reservoirs contained 10 mM histidine, pH 7.0, 50 mM KCl, 1×10^{-4} M $MgCl_2$, 4×10^{-4} M EGTA, 4×10^{-4} $CaCl_2$, 3×10^{-5} M ANS^-. The experiment was carried out at 23 °C

Table 10. Effect of K^+ concentration on the valinomycin-induced enhancement of active uptake

$[K^+]$ (mM)	No Valinomycin		Valinomycin		Fluorescent Amplitude ratio (val/no val)
	Fluorescent $t_{\frac{1}{2}}$	Fluorescent Amp	Fluorescent $t_{\frac{1}{2}}$	Fluorescent Amp	
5	20±1	2.55±0.05	3.5±0.5	2.7 ±0.1	1.06±0.02
50	20±1	2.1 ±0.1	3.5±0.1	2.4 ±0.2	1.14±0.16
100	22±3	1.55±0.05	4.5±0.5	2.05±0.05	1.32±0.08
200	32±3	1.3 ±0.1	5.5±0.5	1.75±0.05	1.35±0.15
400	40±5	1.00±0.05	8.5±0.05	1.42±0.05	1.42±0.17

The basic medium consisted of 0.6 M sucrose, 1×10^{-4} M Mg^{2+}, 4×10^{-4} M Ca-EGTA, 3.17×10^{-5} M ANS^-, 6×10^{-6} M valinomycin and 10 mM histidine, pH 7.0. Mg-ATP and SR were rapidly mixed to final concentrations of 2.5 mM and 0.1 mg/ml, respectively.

progress of the reaction is monitored by multiple sweeps of the oscilloscope trace. In the absence of valinomycin, the rate of ANS^- equilibration in response to Ca^{2+} uptake is slow (Haynes & Chiu, 1977, 1978), resulting in a slower fluorescence increase as compared to Fig. 11*B*. Addition of valinomycin results in an amplitude about 30% larger than the amplitude obtained in the absence of valinomycin.

Experiments were designed to test for the effect of the K^+ concentration and of the valinomycin concentration on the size of the valinomycin-induced increase. These experiments were carried out using high Mg-ATP levels to ensure a constant phosphate potential throughout the experiment. Table 10 shows the valinomycin effect as a function of the KCl concentration. The effect increases with increasing KCl concentration. Table 11 shows the effect of valinomycin concentration. The half-maximal effect is seen between 6.7×10^{-7} and 2×10^{-6} M. With reference to Table 5

Table 11. The effect of valinomycin concentration on its enhancement effect on active uptake

Valinomycin (M)	$t_{\frac{1}{2}}$	Amp
0	40±5	1.2 ±0.1
2.2×10^{-7}	20±2	1.2 ±0.1
6.7×10^{-7}	16±2	1.35±0.10
2.0×10^{-6}	11±1	1.5 ±0.1
6.0×10^{-6}	8±2	1.6 ±0.1

The basic medium consisted of 0.6 M sucrose, 1×10^{-4} M Mg^{2+}, 4×10^{-4} M Ca-EGTA, 3.17×10^{-5} M ANS^-, 400 mM, KCl, and 10 mM histidine, pH 7.0. Mg-ATP and SR, preincubated with valinomycin, were rapidly mixed to final concentrations of 2.5 mM, 0.1 mg/ml and the concentrations indicated, respectively.

of the previous paper (Chiu & Haynes, 1980) it is shown that the half-maximal effects on the rates of passive KCl permeation also occur in this concentration range. The valinomycin concentration used throughout the study (6×10^{-6} M) is sufficient to give maximal Ca^{2+} uptake. Thus our results are typical of the ATPase functioning under optimal conditions with respect to KCl permeation. It would seem that the effects of valinomycin are due to the restoration of KCl depleted by the pump's action as an active $2K^+$ for Ca^{2+} exchanger. A quantitative and mechanistic basis for this conclusion will be given in the Discussion section.

Discussion

The results presented in this paper indicate that active Ca^{2+} transport by SR can be followed rapidly, continuously, and accurately by the ANS^- method. ANS^- can be used as a Ca^{2+} indicator due to its ability to respond to changes in the electrostatic potential of the inner membrane surface resulting from Ca^{2+} accumulation. An increase in fluorescence indicates the build-up of the internal free Ca^{2+} concentration. Conversion of Ca^{2+} transport-induced fluorescence amplitude to the free internal Ca^{2+} concentration show that under optimal conditions the latter can reach values as high as 70±20 mM. Under our conditions we calculate the external Ca^{2+} concentration as 25 μM; the $[Ca^{2+}]_i/[Ca^{2+}]_o$ ratio is therefore 2.8×10^3.

Although we have shown the ANS^- fluorescence signal to be a faithful, albeit nonlinear, measure of changes in the internal free internal Ca^{2+} concentration, on a subsecond time the determination of the total amounts of Ca^{2+} taken up and of pump turnover rates is less direct. Our calculations showed that under optimal conditions, the pump produced an in-

ternal free Ca^{2+} concentration of 30 mM within 0.6 sec. This corresponds to an average rate ($\Delta[Ca]_i/t_{\frac{1}{2}}$) of 50 mM/sec. Multiplication of this by the aqueous volume per mg protein (1.3 ml/mg) would give the number of Ca^{2+} moved per mg protein per sec, the conventional measure of uptake rates when the millipore filtration or external aqueous indicator techniques are used. The result of this calculation is 68 nmol/mg sec. However, the proposed procedure would underestimate the actual amount of Ca^{2+} moved since the inwardly-oriented translocator and the low-affinity sites on the Ca^{2+}-ATPase and negatively-charged phospholipids have substantial binding capacities. In the previous paper (Chiu & Haynes, 1980) we presented calculations which showed that the capacity of the pump would be 20 nmol/mg or 15 mM referred to the internal aqueous phase and that the capacity of the low affinity binding sites was ca. 35 nmol/mg or 27 mM when referred to the internal aqueous phase. The latter capacity would be almost completely saturated at the $t_{\frac{1}{2}}$; the degree of saturation of the inwardly-oriented translocator will be discussed later in this section. Including one or both of these factors would revise our estimate of the average rate to 126–159 nmol mg^{-1} sec^{-1}. This can be compared with the initial rate of the transport reaction of 60–70 nmol mg^{-1} sec^{-1} measured by Inesi and Scarpa (1972) in unfactionated SR using murexide as an external Ca^{2+} indicator. The difference may be due to differences in reaction conditions, type of SR, or the approximations we have used to make our estimates. Our estimate corresponds to a pump turnover number of 6.3–7.9 sec^{-1}. Our estimates are compatible with those of Verjovski-Almeda and Inesi (1979), who determined turnover numbers of 5–6 sec^{-1} for 50 μM ATP and 10–12 sec^{-1} for millimolar ATP.

Applying our estimates of the turnover numbers to the mechanism of Fig. 1, we can conclude that all unimolecular rate constants (and biomolecular rate constants multiplied by substrate or cofactor concentrations at saturating concentrations) must be greater than or equal to 7.1 ± 0.8 sec^{-1}. Under less than optimal conditions, the initial rate is limited by the substrate or cofactor concentrations. As the transport reaction proceeds the rate decreases and finally approaches zero as the maximal steady-state uptake is achieved. In this state the carrier is inhibited by saturation of its Ca^{2+} site with internal Ca^{2+}. All of the observations made in the present study can be rationalized in terms of the mechanism of Fig. 1. In the remainder of the paper we will discuss this mechanism, important mechanistic observations made by other workers, and their relation to our data on a step-by-step basis. The analysis will show that the phenomenon of two phases of uptake can be explained as partial and multiple turnovers (respectively). It will also be shown that the accelerative effect of high K^+ concentrations results from facilitation of the unloading of two inwardly-oriented translocator by a competition mechanism.

1) Steps 1–4: Loading of the Carrier with Ca^{2+} and Mg-ATP

Since the early observations of Hasselbach and Makinose (1963), a multitude of studies has shown that the Ca^{2+} transport system is activated by very low Ca^{2+} concentrations. We are not aware of any evidence for nonrandom addition of Ca^{2+} and Mg-ATP to the pump, and the existing kinetic information can be explained by random order of addition of these substrates. The existence of a second "regulatory" Mg-ATP site or of variable affinity of the Mg-ATP binding site must be considered (*cf.* Neet & Green, 1977). This would require modification of Fig. 1. Also, our study shows that long-term deprivation of Ca^{2+}, where the carrier remains in the unloaded form for a number of minutes, can result in altered carrier kinetics. The data of Fig. 10 and Table 9 show that the occupation of the high affinity Ca^{2+} binding sites is critical to the stability of the transport system. When the free Ca^{2+} concentration was lowered to about 10^{-7} M, the transport system was found to be unstable and exhibited a delayed release phenomenon. This unstable state persists even when the Ca^{2+} level is brought back to the optimal saturating level (29 μM) within the 3-msec mixing time of the stopped-flow experiment. The time required to restore the total stability of the transport system is between 2 and 45 sec. From high affinity binding studies (Meissner, 1973; Ikemoto, 1974; Chiu & Haynes, 1977, Inesi et al., 1978*a*, 1978*b*) carried out in the presence and absence of Mg^{2+}, we can conclude that reducing the free Ca^{2+} concentration to 10^{-7} M should render the high affinity site of the pump less than 10% occupied with Ca^{2+}. It would seem that prior occupation of this site is necessary for maximal rates of transport and the stability of the gradient established by the pump. Similar findings have been reported based on behavior of the phosphoenzyme (Froehlich, 1978; McIntosh & Berman, 1978).

We interpret the requirement of prior addition of Ca^{2+} to mean that in the absence of Ca^{2+}, the enzyme can exist in a conformation or state of aggregation state (*cf.* Froehlich & Taylor, 1975, 1976; Vanderkooi et al., 1977) which is not conductive to transport. Conversion from the inactive to the active form upon binding in the absence of Mg-ATP could require an incubation time possibly as long as 45 sec. In the presence of Mg-ATP the conversion was not accom-

plished in 100 sec, the approximate lifetime of our active uptake experiment. Therefore, Fig. 1 should be amended to include the reaction

$$E \rightleftharpoons E^* \text{ (slow)} \quad (5)$$

where E^* is a conformationally or aggregationally altered form of the enzyme which cycles through steps *1–10* more slowly than *E*. Froehlich (1978) has given evidence for two conformational states of the enzyme whose relative abundance is controlled by the concentrations of Mg^{2+} and Ca^{2+} in the medium. Prior incubation with high (20 mM) Mg^{2+} in the absence of Ca^{2+} was shown to decrease the Ca^{2+}-stimulated rate of phosphorylation. Addition of 10 μM Ca^{2+} in the preincubation medium restored the high rates. Other information supporting the existence of two conformational states of the enzymes is also supplied by the spectroscopic studies of Champeil et al. (1976) and Croan and Inesi (1977) and from studies of SH group reactivity (Murphy, 1978).

A number of studies have shown that the Mg-ATP complex is the true substrate for the ATPase (Vianna, 1975; Yamamoto & Tonomura, 1967). Our present study (Table 1, Fig. 7) further supports this observation. The initial Ca^{2+} transport rate, the overall transport rate ($1/t_{\frac{1}{2}}$) and the net Ca^{2+} accumulation increase with increasing Mg^{2+} concentration. Slow Ca^{2+} transport ($t_{\frac{1}{2}}=25$ sec) without added Mg^{2+} was probably the result of endogenous or contaminating Mg^{2+} in the reaction medium (Table 7). Since the phosphoenzyme level remains constant in the absence or presence of Mg^{2+} (Garrahan, Rega & Alonso, 1976) both the phosphorylation and dephosphorylation reactions are involved in the stimulatory effect we observed. At a higher Mg^{2+} concentration, 5 mM and greater, a distinct rapid burst of Ca^{2+} uptake with a 50-msec duration was seen (Fig. 7). The analysis shows that this represents a partial turnover of the pump.

The inhibitory effect of high Ca^{2+} concentrations (25 to 100 μM) on the initial rate and overall transport rate can also be explained in terms of step *1*. We have considered several possibilities for explaining this (Makinose & Hasselbach, 1965; Weber et al., 1966; Inesi et al., 1970; Kanazawa et al., 1971; Panet, Pick & Selinger, 1971) and conclude that it is due to the formation of an inhibitory Ca-ATP complex analogous to the product of step *1*. We have observed (experiments not shown) that when excess Ca-ATP is added to the uptake medium during the course of active Ca^{2+} uptake, the progress of the uptake can be stopped within 3 msec, the stopped flow rapid mixing time. The addition of the inhibitory complex results in a slow leak of Ca^{2+}. Kanazawa et al. (1971) showed that $E\sim{}^{32}P$ formation from the SR and $AT^{32}P$ can be stopped rapidly resulting in exponential decay of $E\sim{}^{32}P$ without any appreciable lag phase when unlabeled ATP was added to the external SR medium. Their suggestion was that the Mg-ATP binding site is located on the outside surface of the membrane. Our results on Ca-ATP addition are in agreement with their conclusion. Also, the Mg-ATP binding site should be located on the outside surface since high bimolecular rate constants for the binding of this substrate can be estimated.

2) Step 5: Phosphorylation of the Enzyme
Step 6: Translocation of the Active Site

The phosphorylation of the enzyme (step *5*) has been documented in a large number of studies (Yamamoto & Tonomura, 1967; Kanazawa et al., 1971; Boyer et al., 1977; Bastide et al., 1973; Froehlich & Taylor, 1975, 1976). Data on the rate of $E\sim P$ formation at optimal Ca^{2+} and Mg-ATP concentrations at 25 °C gives $k_5=85$–$150\ sec^{-1}$ (Froehlich & Taylor, 1975; Inesi et al., 1978*a*, 1978*b*; Verjovski-Almeida & Inesi, 1979).

In step 6, Ca^{2+} is translocated across the membrane (*cf.* Inesi et al., 1978*a*, 1978*b*). Through this step, or possibly through the subsequent step, the active site loses its high affinity for Ca^{2+}; the affinity decreased by a factor of 10^3. Inesi et al. (1978*a*, 1978*b*) and Verjovski-Almeida & Inesi (1979) have performed EGTA quench-flow experiments in which the kinetics of step *6* are resolved. The experiments are based on their reasoning that the translocation step is the first step which makes Ca^{2+} inaccessible to EGTA in the external medium. Their quench-flow experiments revealed a burst of Ca^{2+} uptake which probably corresponds to the first partial turnover of the pump. Using ITP which is broken down at slower rates than ATP, they were able to show that the initial burst of Ca^{2+} uptake precedes the dephosphorylation of $E\sim P$, in agreement with the order of reaction given in Fig. 1. Comparison of their experiments on the kinetics of Ca^{2+} inaccessibility to the outside and our experiments on the kinetics of Ca^{2+} on the inside shows that the two processes are nearly simultaneous. The time for removal of 5 nmol/mg protein is 20 msec. Our experiments show that Ca^{2+} is released to a free concentration of ca. 2 mM inside, in an initial burst of 50 msec duration (*cf.* Table 3).[3]

[3] Our findings of a turnover number of $7.1\pm0.8\ sec^{-1}$ under active transport conditions predicts that steps *1–6* must take ca. 140 msec of less. The Ca^{2+} must be released to the internal aqueous medium in order for it to shield the negative surface charge of the internal surface. This view is supported by our studies of ANS^- binding (Chiu et al., 1980) which indicate that the major portion of the ANS^- fluorescence signal comes from ANS^- bound to phospholipids.

This corresponds to 2.6–20.6 nmol/mg transported Ca^{2+}, or 0.26–2.06 Ca^{2+} moved per pump.[4] With reference to Fig. 1 this can be interpreted as meaning that either Ca^{2+} must dissociate rapidly from the $E\sim P$ Ca^{2+} complex or that the dephosphorylation step (7) with attendant Ca^{2+} dissociation must be rapid ($t_{\frac{1}{2}} < 30$ msec). (In the scheme of Inesi et al., 1978*a*, 1978*b*, steps *6* and *7* were combined). A study of *S–H* group reactivity (Murphy, 1978) has shown that phosphorylation of the enzyme brings about an extensive structural change. Assuming that the fast phase represents steps *6* and *7*, our turnover number allows us to estimate $k_6 \geqq 13.8\ \text{sec}^{-1}$ and $k_7 \geqq 13.8\ \text{sec}^{-1}$.

The observation of a fast phase of Ca^{2+} release to the inside (10^{-4} M Mg^{2+}) when the internal K^+ concentration was high (50 mM) but not when it was low (10 mM) can be readily explained as K^+/Ca^{2+} competition for the inwardly-oriented translocator. At the lower K^+ concentration, the bulk of the Ca^{2+} remains bound to the translocator and is not set free in the lumen. (Comparison of the upper limit of a fast phase amplitude with the fast phase amplitudes observed in the passive Ca^{2+} jump experiments (Chiu & Haynes, 1980) rules out binding to other surface elements as an explanation for the lack of an effect). If the K^+/Ca^{2+} competition mechanism is accepted, then we can conclude that the dissociation constant for $E\sim P$ Ca_2 is significantly less than 2 mM (the free concentration which would obtain if the Ca^{2+} dissociated from the carrier and equilibrated with the low affinity internal finding sites). From the lack of dissociation, we can estimate an upper limit of ca 0.3 mM (Table 1) for K_d value of $E\sim P$ Ca_2 treating it as if it were $E\sim P$ Ca_2. Measurements of Inesi et al. (1978*a, b*) on the effect of internal Ca^{2+} concentration on the electron spin resonance spectrum of a spin-labeled Ca^{2+}-ATPase yielded an estimate of 10^{-3} M for this K_d value at high KCl concentration. Our observation that millimolar free concentrations of internal Ca^{2+} can be obtained in the fast phase in the presence of 5–400 mM K^+ indicates that the K_d of $E\sim P$ K must lie in this range (50–400 mM).

The analogous effect of high Mg^{2+} to produce a fast phase can also be explained by Mg^{2+}/Ca^{2+} competition. The data of Table 1 and Fig. 7 taken at low K^+ concentrations show that a fast phase is not observed at 0.1 mM Mg^{2+} but is observed at 5 mM Mg^{2+}. The phase gave rise to an estimated internal free Ca^{2+} concentration of 2 mM. This result could be consistent with Mg^{2+}/Ca^{2+} competition for occupation of the inwardly oriented $E\sim P$, but the result is also open to an alternative interpretation involving the role of Mg^{2+} to catalyze the breakdown of $E\sim P$ to $E\cdot P$. When both the K^+ and Mg^{2+} are at optimal concentrations, no distinct rapid phase is seen. Under this condition, step *7* is no longer limiting to the overall transport and the enzyme continues to cycle at rates comparable to its initial rate. The turnover number for the first partial cycle of the enzyme was estimated as ca. $13.8\ \text{sec}^{-1}$, and the average turnover number for the first several cycles at optimal internal K^+ and Mg^{2+} concentrations is close to this value (ca. $7.1\ \text{sec}^{-1}$). This comparison would identify step *6* as the rate-limiting step under optimal conditions.

3) Step 7: Dephosphorylation of the Enzyme and the Role of Internal Mg^{2+}

The dephosphorylation step is necessary to drive the enzyme through its cycle. Phosphoenzyme studies (Kanazawa et al., 1971; Froehlich & Taylor, 1975, 1976) have shown that the reaction is catalyzed by Mg^{2+}. We have observed that the overall rate of the transport reaction increases with increasing Mg^{2+} concentrations. Part of this effect is due to the formation of Mg-ATP, and part of it may be due to a catalytic effect of free Mg^{2+} on the inside or outside surface. At between 1 and 5 mM Mg^{2+} we observed a catalytic effect which cannot be attributed to the formation of Mg-ATP. Using the published binding constant of Mg^{2+} with ATP ($1.1\times10^4\ \text{M}^{-1}$, Walaas, 1958), we calculate that over 99% of the ATP is converted to Mg-ATP. However, we observed a greater than 12% increase in the initial rate of fluorescence charge when the Mg^{2+} concentration was increased from 1 to 5 mM (Table 1), pointing to the involvement of a Mg^{2+} site. The Mg^{2+} jump experiments give strong evidence that the catalytic site is not exposed to the outside medium and probably is exposed to the inside surface. The Mg^{2+} enhancement effect on the rate is absent if the cation is added at the same instant that the ATP is added. This proves that the Mg^{2+} requires more than 27 sec, the half-time of the active transport reaction under these conditions, to reach its catalytic site. Since the half-time for passive equilibration of Mg^{2+} across the membrane of close to 200 sec (Chiu & Haynes, 1980), these observations suggest that the site may be located on the inside surface.[5] The enhancement effect seen with 10^{-4} M Mg^{2+} is attributable to a catalytic effect

[4] The lower limit is estimated using 1.3 µl/mg aqueous volume and assuming no Ca^{2+} binding to internal sites. This upper limit is calculated assuming this binding to occur with capacities and affinities used in the previous paper (Chiu & Haynes, 1980).

[5] An alternative explanation is that the site is primarily accessible from the outside surface but has slow binding kinetics or that the Mg^{2+} effect involves slow conformational or aggregational changes. This could be checked by repeating the experiments mea-

on the reactions as opposed to its being a countertransported species. The Mg^{2+} concentration is too low to be useful as a countertransported species (it would support only $1/150^{th}$ of a pump turnover[6]), and the ANS^- method will not detect a divalent cation for divalent cation exchange. Furthermore, if the Mg^{2+} were countertransported, it should be swept from the SR interior after the first turnover and the subsequent turnovers should occur at very slow rates. Since this is contrary to our findings, we conclude that the Mg^{2+} is not significantly countertransported during the lifetime of our experiment. This conclusion is supported by the results of Ueno and Sekine (1978) which show that active transport does not suppress the internal Mg^{2+} concentrations to an extent which is compatible with a one-to-one obligatory Mg^{2+}/Ca^{2+} exchange mechanism.

There is a wealth of information showing a catalytic influence of Mg^{2+} on the rate of dephosphorylation of the enzyme (Kanazawa et al., 1971; Froehlich & Taylor, 1975, 1976). Using Mn^{2+} as a competitor, Kalbitzer, Stahlik and Hasselbach (1978) discovered four Mg^{2+} binding sites with moderate affinity constants ranging from 2×10^2 M^{-1} to 6×10^3 M^{-1}, and a high affinity Mg^{2+} binding site for which Mn^{2+} does not compete. Garrahan et al. (1976) reported that some Mg^{2+} bound becomes unavailable for exchange with free Mg^{2+} in the medium when the enzyme is phosphorylated. Calorimetric studies (Y. Epstein and R. Biltonen, unpublished) have shown evidence for $\overset{E}{\underset{Mg}{*}}$ and $\overset{E\,P}{\underset{Mg}{*}}$ complexes. Mg^{2+} has also been shown to serve as a catalytic factor in the enzyme-mediated P_i-H_2O oxygen atom exchange reaction (Boyer et al., 1973). The model of Fig. 1 must be modified to take into account the binding of Mg^{2+} to the enzyme. Although a number of complicated models involving association and dissociation could be envisioned, the assumption that Mg^{2+} remains on the enzyme throughout the cycle is adequate to explain the present observations.

As noted in the previous section, we believe that high concentrations of internal K^+ are capable of displacing Ca^{2+} from $\overset{E\sim P.}{\underset{Ca_2}{\downarrow}}$ At low Mg^{2+} concentrations (10^{-4} M) a rapid phase of Ca^{2+} release to the interior is observed, followed by a slower phase representing a number of pump turnovers whose rates are limited by step *7* of Fig. 1. At higher Mg^{2+} concentrations, this rate limitation is removed. The elevation of the maximal free internal Ca^{2+} concentration achieved by the pump would be the result of an increase of the fraction of the pump in the $E \cdot P$ state relative to the $E \sim P$ state, and the lower Ca^{2+} affinity of the former (*cf.* Inesi et al., 1978*a*, *b*; Verjovski-Admeida & Inesi, 1979).

The literature contains suggestions that K^+ serves to increase the rate of dephosphorylation of $E \sim P$. Due to the nature of these experiments, it is not possible to determine whether the locus of action was step *7* acting as a catalyst or on step *9* acting as a countertransported "reactant". Recent work of Shigekawa and Dougherty (1978*a*) reports two acid stable forms $E \sim P$ and suggests that the K^+ effect is distal to the Mg^{2+}-catalyzed step. In a companion paper (Skigekawa & Dougherty, 1978*b*) they reported that the effect of ATPase activity showed a first-power dependence on the K^+ concentration which would be consistent with a catalytic effect on step *7*. These observations are also consistent with a stimulatory effect through the mechanism of step *9*.

4) Steps 8 and 9: Dissociation of Ca^{2+} and Association of 2 K^+ with the Translocator

Dissociation of the Ca^{2+} from the inwardly-oriented translocator (step *8*) is required by all plausible models of the pump function. The energy supplied by the ATP for uphill Ca^{2+} transport can be used to produce differences in the affinities of the inwardly- and outwardly-oriented forms of the translocator for Ca^{2+}. Alternatively, the enzyme phosphorylation could result in a shift in the orientational equilibrium of the translocator, in favor of the inward orientation. We believe that both types of energetic contribution must be involved. The conformational changes in steps *5–7* will have reduced the affinity of the translocator for Ca^{2+} to a much lower level. The highest concentration of internal Ca^{2+} obtainable in active transport will ultimately be determined by the K_d of the inwardly-oriented $E \cdot P$ for Ca^{2+}. The model of Fig. 1 allows the apparent K_d of $E \cdot P$ to be lowered still further by the mechanism of K^+/Ca^{2+} competition. Electrogenic models of carrier function, which do not allow the translocator to return in the loaded state, do not have this energetically important feature. Our findings of enhancement of the net transport

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suring ATPase activity in the presence of a Ca^{2+} and Mg^{2+} ionophore. The interpretation would not be unambiguous since one would have to assume that the ionophore could not deliver Mg^{2+} directly to a buried active site of the enzyme. Garrahan et al. (1976) have shown that the catalytic effect of Mg^{2+} to catalyze the breakdown of the phosphophzyme. This observation was also made for the detergent-solubilized enzyme, indicating that in our experiments the Mg^{2+} binding site should be occluded for at least the first turnover.

[6] Using the pump capacity as 2 moles Ca^{2+}/turnover and the aqueous volume of the internal space as 1.3 µl/mg protein (this study) and the mol wt of the ATPase as 10^5 dalton, we conclude that each pump turnover will increase the internal Ca^{2+} concentration by 15 mM. If obligate Mg^{2+}/Ca^{2+} exchange were operative, then only 10^{-4} M Ca^{2+} (equivalent to $1/150^{th}$ of a pump turnover) could be pumped in.

reaction by internal K^+ are explained directly by the model of Fig. 1. High internal K^+ has the dual effect of (i) removing Ca^{2+} from the inwardly-oriented translocator by the mechanism of competition and (ii) serving as a means of returning the translocator to its outside orientation. Electrogenic models do not predict stimulation by internal K^+ via K^+/Ca^{2+} competition; K^+ binding to the translocator would result in inhibitor since the translocator must return to the outside in the *unloaded* state.

The retardation of the active transport rate observed in our KCl jump experiments (Table 7) is also explained directly by Fig. 1. High levels of *internal* K^+ or other monovalent cations are necessary for maximal rates of transport to be observed, since K^+ counter-transport is required to return the translocator to the outside. Electrogenic models for pump function predict the exact opposite of the results observed in our KCl jump experiments. They predict that high concentrations of KCl outside and low concentrations inside would *increase* the rate of pump function, since the KCl jump would result in a negative-inside membrane potential due to the high Cl^- permeability and gradient. The requirement for internal K^+ is easily understood in terms of the model of Fig. 1. In the model the requirement for countertransport of two monovalent cations is absolute. At low internal K^+ concentrations the overall reaction is limited by K^+ depletion of the lumen. Replenishment occurs via the passive $K^+(Cl^-)$ permeability of the vesicles which we characterized in the previous paper (Chiu & Haynes, 1980). For the one-third of the vesicles which are poorly permeable to KCl, the K^+ depletion imposes severe limitations on the Ca^{2+} transport. This is the basis of the valinomycin enhancement effect presented in the results and discussed in the next section.

The observations made here allow us to rule out Mg^{2+} as a major countertransport ion. The catalytic effect of low internal concentrations of Mg^{2+} has been discussed already. Active uptake initiated in the presence of 5 mM Mg^{2+} and 5 mM K^+ (Fig. 7) showed two phases of fluorescence increase. If the Mg^{2+} has been preferentially counter-transported then a lag would have been observed, since a Mg^{2+}/Ca^{2+} exchange would not change the total divalent cation concentration in the SR lumen and would not change the total level of fluorescence. We conclude from this that even at this high ratio of Mg^{2+} to K^+ concentration, the Mg^{2+} is not preferentially countertransported. This is in agreement with the findings of Ueno and Sekine (1978) showing that active transport does not suppress the internal Mg^{2+} concentrations to the extent predicted by obligate 1:1 countertransport. The dissociation constant for Mg^{2+} in step *9* must be greater than 5 mM.

In a previous section we showed that the K_d for $\underset{Ca}{E\sim P}$ is less than 0.3 mM, and that the translocator is susceptible to K^+/Ca^{2+} competition. From the high internal Ca^{2+} concentrations built up by the pump at the end of net transport, one must conclude that the K_d of $\underset{Ca}{E\cdot P}$ is much larger than 1 mM. The maximal internal Ca^{2+} concentration is ca. 17 mM at low (0.1 mM) K^+ concentrations and is ca. 64 mM at high (100 mM) K^+ concentrations. This is explained by the K^+/Ca^{2+} competition expressed in step 8 of Fig. 1. The apparent K_d values for $\underset{Ca}{E\cdot P}$ could be a substantial fraction of the observed values of Ca^{2+} concentration at maximal steady-state uptake. This is in apparent agreement with our finding that the K_m for passive efflux of trapped Ca^{2+} is greater than 5 mM (Chiu & Haynes, 1980). A low affinity of $E\cdot P$ for Ca^{2+} is necessary for tight coupling between the ATPase and Ca^{2+} transport functions of the pump. At the end of the transport reaction, trans-inhibition sets in and the production and return of the K^+-laden form of the translocator (steps *9* and *10*) become rate-limiting.

5) Step 10: Return of the Carrier

Under optimal conditions the progress curve for the fluorescence increase indicates a rapid transport reaction which cannot be resolved into individual phases. Such behavior is observed when the K^+ concentration is sufficient to guarantee unloading of the carrier on the inside surface and when the Mg^{2+} concentration is sufficient to guarantee maximal rates of dephosphorylation. The lack of an initial burst under these conditions indicates that the rate of return of the carrier (step *10*) is greater or equal to the rate of the initial crossing of the energized carrier (step *6*). Thus it must have a rate constant greater than or equal to 7.1 sec^{-1}, the maximal average turnover number calculated under optimal conditions of activation. This value was probably somewhat affected by trans-inhibition of the pump; since a similar value was calculated for k_6 (13.8 sec^{-1}) it would seem that step *10* does not impose a rate limitation to the overall process during the first complete turnover. Our previous study (Chiu & Haynes, 1980) gave evidence that the conformational energetics of the unphosphorylated form of the enzyme favor outward orientation of the translocator. If the $E\cdot P$ form of the enzyme behaves similarly, the reverse reaction (step -10) would be expected to be much slower than the forward reaction (step *10*). This would offer an explanation for the slow rate of pump reversal observed upon withdrawal of external Ca^{2+} after active transport.

6) Unloading the Carrier of Countertransported Ions and P_i

The dissociation constants of K^+ and Mg^{2+} for the outwardly-directed $E \cdot P$ state must be high since these cations do not give marked inhibition of the transport reaction. Inhibition is expected from high levels of P_i in keeping with this ion's role as a product of the reaction.

7) Consideration of Possible Electrogenicity of the ATPase

The present study has already given two major lines of evidence that the pump does not operate electrogenically: (i) The effect of high K^+ concentration to speed up the net transport reaction is not explained by electrogenic models but is explained by the countertransport model. (ii) The acceleration of Ca^{2+} transport by a KCl jump, predicted by electrogenic models, is not seen. The finding that slower rates are observed, compared to the symmetrical KCl case, is fully explained by the countertransport model. In this section we will present a third line of evidence against the electrogenic model: (iii) Addition of valinomycin, at concentrations sufficient to unmask an electrogenic transport mechanism in the KCl impermeable vesicles, does not increase the contributions of these vesicles to the total Ca^{2+} uptake.

A valinomycin effect was used as an argument for an electrogenic model for the Ca^{2+}-ATPase action by Zimniak and Racker (1978). They analyzed the rate of Ca^{2+} uptake of the reconstituted ATPase using the phosphate precipitating system. The latter was necessary for appreciable degrees of transport to be observed. Their criterion for electrogenicity was that the introduction of valinomycin, a known charge carrier, served to increase the Ca^{2+} uptake rates observed. The implication of this reasoning is that the ionophore could not be exerting its influence on the uptake rates by functioning in noncharge-carrying modes such as KCl translocation. Our previous study showed that valinomycin can translocate KCl in an electrically silent reaction. This raises serious questions about the interpretations given in the study (Zimniak & Racker, 1978). Their study also reported effects of a "K^+ gradient," but these were weak, giving rise to only a twofold variation in rate for an enormous variation in inside to outside K^+ concentration ratio. Furthermore, the stimulatory effect of valinomycin was not seen at low K^+ concentrations although the authors' interpretation (electrogenic pump function in the absence of adequate charge-carrying passive permeability) should require a stimulatory effect at all concentrations.[7]

The effects of valinomycin on the rate and extent of Ca^{2+} uptake observed in the present study can be explained on the basis of the ionophore's ability to facilitate the influx of KCl, both by its activity as a K^+ translocator in the presence of existing anion permeability mechanisms and by its intrinsic activity as a KCl translocator through its effect on the KCl impermeable fraction of the SR. In the previous study (Chiu & Haynes, 1980) we showed that valinomycin can increase the KCl permeability of the SR by both mechanisms. Evidence that val-K^+ can ion pair with anions and translocate ion pairs is ample (Pressman & Haynes, 1969; Haynes & Pressman, 1974; Haynes & Simkowitz, 1977; Ginsburg, Tosteson & Tosteson, 1978). The lipid bilayer study of Ginsburg et al. (1978) showed that trinitrocresol (TNC^-) can increase valinomycin-induced conductance, but will also increase the rate of cation isotope flux to a sevenfold greater extent than can be accounted for by the conductance. This is explained by the contributions of ion-pairs of the val-M^+ and TNC^- to the overall M^+ translocation (i.e., movement of M^+-TNC^-). The ion pairing reactions (Haynes & Simkowitz, 1977; Ginsburg et al., 1978) are quite avid with lipophilic anions, but are also expected to a smaller extent with Cl^-.

Quantitative and mechanistic consideration of the effect of valinomycin provides evidence that the pump does not function electrogenically. The argument can be made with reference to Fig. 12 which shows the expected effects of valinomycin for the KCl-permeable vesicles (*I*) and KCl impermeable vesicles (*II*). The previous study (Chiu & Haynes, 1980) gave evidence that, in the presence of electrically-active anion permeability, valinomycin makes its greatest contribution by translocating K^+. Thus Fig. 12-*I* considers only the electrically-active mode of valinomycin action. Our previous study showed that in the absence of an electrically-active anion permeability the major contribution made by valinomycin to KCl permeability is electrically neutral KCl movement. Thus Fig. 12-*II* considers the contribution of the electrically-neutral mode of action for the case where the pump acts in the exchange-diffusion mode (*II-A, B*). For case

[7] The authors also based their arguments on the effects of experimental manipulations on the fluorescence of ANS^- which they considered to be a membrane potential probe. We have shown that ANS^- permeates more rapidly as an ion pair with K^+ than it does as a free ion, and is therefore not competent to serve as an indicator of membrane potential (Haynes & Simkowitz, 1977). Furthermore, poor correspondence was observed between the valinomycin stimulatory effects on Ca^{2+} transport and the effects of the putative membrane potential on ANS^- fluorescence.

II it is clear that the electrically-neutral mode of action of valinomycin will not contribute to Ca^{2+} movement driven by the pump operating in the electrogenic mode, so the electrically-active mechanism of valinomycin action is considered (*II-C, D*).

Consideration of Fig. 12 reveals that the major effect of valinomycin would be expected from the KCl impermeable vesicles. The KCl permeable vesicles have sufficient K^+ and Cl^- permeability to allow Ca^{2+} uptake to occur by either mechanism, and the only influence that valinomycin would have is to speed up the rate. In contrast, the KCl-impermeable vesicles would be inhibited in their Ca^{2+} uptake. For the $2K^+$ for Ca^{2+} exchange mode of pump action (*II-A, B*) the inhibition would be due to K^+ depletion. For the electrogenic mode of pump action the inhibition would be absolute. Valinomycin would release the inhibition according to both mechanisms. However, the valinomycin effects on the electrogenic mechanism should be seen at much lower concentrations than required for the valinomycin effect on the exchange mechanism. The reason for this is that the current-carrying mode is ca. 690 times more rapid than the electrically-silent mode (Chiu & Haynes, 1980). The comparison can also be made on the basis of crude estimates of numbers of ions which must be moved during the $t_{\frac{1}{2}}$ of the active transport reaction. The overall $t_{\frac{1}{2}}$ for the fluorescence change accompanying the active transport reaction in the presence of 2×10^{-6} M valinomycin is ca. 11 sec. If this reaction resulted in 70 mM Ca^{2+} in the lumen of the KCl impermeable SR then 140 mM K^+ would have to be removed from the lumen by the valinomycin-facilitated mechanisms of Fig. 12-*II*. Within the $t_{\frac{1}{2}}$ of the active transport reaction, 70 mM K^+ would have to removed. The KCl transport results of the previous study (Chiu & Haynes, 1980) show that valinomycin working in the electrically silent mode can move 200 mM K^+ in 55 sec. It would be expected to move 70 mM K^+ in about 19 sec. This time is close to the experimentally-measured half-time of the active transport reaction (11 sec). Thus model *II-A, B* for the valimomycin enhancement effect is permissible. Under the same conditions our previous study shows that valinomycin operating in the electrically-active mode can move 200 mM K^+ in 200 msec. The time for moving 70 mM K^+ would be 70 msec. This time is $1/157^{th}$ as long as the experimentally observed half time suggesting that the effect should have also been seen at 1.3×10^{-8} M. In fact, the valinomycin effect was not observed below 6.7×10^{-7} M, a concentration 51 times as large. Although the choice of numbers and the simplifications made to produce these estimates make the final values rather imprecise, we consider the discrepancy between the predictions of model *II-C, D* and the results to be great enough for the electrogenic model to be ruled out. A more clear-cut demonstration will have to await either the isolation and full characterization of the KCl impermeable SR or the reconstitution techniques leaving the Ca^{2+}-ATPase capable of producing Ca^{2+} gradients of the magnitude seen here.

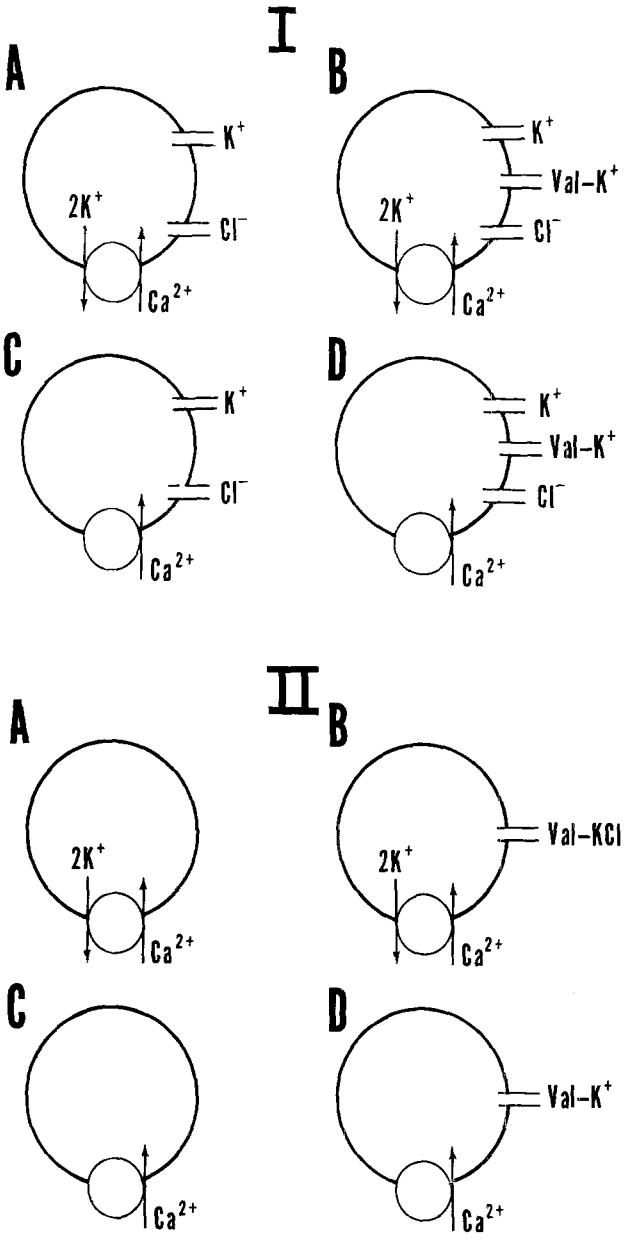

Fig. 12. Mechanisms by which valinomycin can facilitate Ca^{2+} transport. Mechanisms for KCl permeable vesicles and impermeable vesicles are given in sections *I* and *II*, respectively. Mechanisms *A* and *B* are for the Ca-ATPase operating as a $2K^+$ for Ca^{2+} exchanger. Mechanisms *C* and *D* are for the enzyme acting as an electrogenic Ca^{2+} pump. Other details of the mechanisms are discussed in the text

Conclusions

The results of the present study and the studies by other investigators cited here lead to the following picture of the Ca^{2+}-ATPase function. The free energy of hydrolysis of ATP as translated into a 2800-fold Ca^{2+} gradient by an energy transduction process in which both the orientation of the translocator and its affinity for Ca^{2+} are changed. These alterations are brought about by the phosphorylation of the enzyme. The enzyme in its unphosphorylated state has a high affinity for Ca^{2+} on its translocator site and has conformational energetics favoring an outward translocator orientation. Phosphorylation of the enzyme makes the outwardly-oriented form unstable and brings about an inward orientation. This process, which occurs with a unimolecular rate constant of 13.8 sec^{-1}, is the rate-limiting step in the transport sequence when all other conditions are optimal. After reorientation, the translocator experiences a ca. 1000-fold decrease in affinity for Ca^{2+}. If the internal alkali cation concentration is high, the Ca^{2+} is dissociated immediately. If it is low, the dissociation must await dephosphorylation, which further decreases the Ca^{2+} affinity of the translocator. The latter process is facilitated by Mg^{2+} in the lumenal aqueous space. Dephosphorylation of the enzyme makes the inward orientation of the translocator unstable. Return of the translocator to an outward orientation requires the binding and countertransport of two alkali cations or Mg^{2+}. Analysis shows that the inwardly-oriented translocator has a higher affinity for K^+ than for Mg^{2+}. Thus K^+ is the major countertransported ion. Binding for this species assists in lowering the apparent affinity of the inwardly-oriented translocator for Ca^{2+}, thus increasing the ability of the pump to establish a gradient.

The obligate cation exchange feature of the pump, advanced here, suggests one important structural feature of the translocator: a double negative charge on or about the Ca^{2+} binding site. The double negative charge would supply a portion of the binding energy necessary to obtain the high Ca^{2+} affinity of the outwardly-oriented translocator. It would also result in a neutral Ca^{2+}-translocator complex capable of crossing hydrophobic barriers with minimal electrostatic interactions. A decrease in the affinity of the translocator would be brought about by rearrangement of the liganding groups. A reduction in the divalent *vs.* monovalent specificity would be brought about by segregation of the negatively-charged groups. Obligate cation exchange results from the necessity of charge neutralization for reorientation of the translocator.

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References

Åkerman, K.E.O., Wolff, C.H.J. 1979. Charge transfer during Ca^{2+} uptake by rabbit skeletal muscle sarcoplasmic reticulum vesicles as measured with Oxanol VI. *FEBS Lett.* **100**:291

Bastide, F., Meissner, G., Fleischer, S., Post, R.L. 1973. Similarity of the active site of phosphorylation of the adenosine triphosphatase for transport of sodium and potassium ions in kidney to that for transport of calcium ions in the sarcoplasmic reticulum of muscle. *J. Biol. Chem.* **218**:8385

Beeler, T., Martonosi, A. 1979. The relationship between membrane potential and Ca^{2+} fluxes in isolated sarcoplasmic reticulum vesicles. *FEBS Lett.* **98**:173

Beeler, T., Russell, J.T., Martonosi, A. 1979. Optical probe responses on sarcoplasmic reticulum: Oxacarbocyanines as probes of membrane potential. *Eur. J. Biochem.* **95**:579

Boyer, P.D., Meis, L. de, Carvalho, M., Hackney, D.D. 1977. Dynamic reversal of enzyme carboxyl group phosphorylation as the basis of oxygen exchange catalyzed by sarcoplasmic reticulum adenosine triphosphatase. *Biochemistry* **16**:136

Campeil, P., Bastide, F., Taupin, C., Gary-Bobo, C.M. 1976. Spin-labelled sarcoplasmic reticulum vesicles: Ca^{2+}-induced spectral changes. *FEBS Lett.* **63**:270

Chiu, V.C.K., Haynes, D.H. 1977. High and low affinity Ca^{2+} binding to the sarcoplasmic reticulum. Use of a high-affinity fluorescent calcium indicator. *Biophys. J.* **18**:3

Chiu, V.C.K., Haynes, D.H. 1980. Rapid kinetic study of the passive permeability of a Ca^{2+}-ATPase rich fraction of the sarcoplasmic reticulum. *J. Membrane Biol.* **56**:203

Chiu, V.C.K., Mouring, D., Watson, B.D., Haynes, D.H. 1980. Measurement to surface potential and surface charge densities of sarcoplasmic reticulum membranes. *J. Membrane Biol.* **56**:121

Croan, C.R., Inesi, G. 1977. Ca^{2+}-dependent effect of ATP on spin-labelled sarcoplasmic reticulum. *J. Biol. Chem.* **252**:3044

Duggan, P.F. 1977. Calcium uptake and associated adenosine triphosphatase activity in fragmented sarcoplasmic reticulum. Requirement for potassium ions. *J. Biol. Chem.* **252**:1620

Du Pont, Y. 1979. Electrogenic calcium transport in the sarcoplasmic reticulum membrane. *In*: Cation Flux Across Membranes. Y. Mukohata and L. Packer, editors. pp. 141–160. Academic Press, New York

Ebashi, S., Lipmann, F. 1962. Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J. Cell. Biol.* **14**:389

Froehlich, J.P. 1978. The effect of pretreatment with calcium and magnesium ions on phosphoenzyme formation by sarcoplasmic reticulum. ATPase. *Biophys. J.* **24**:61

Froehlich, J.P., Taylor, E.W. 1975. Transient state kinetic studies of sarcoplasmic reticulum adenosine triphosphatase. *J. Biol. Chem.* **250**:213

Froehlich, J.P., Taylor, E.W. 1976. Transient state kinetic effects of calcium ion on sarcoplasmic reticulum adenosine triphosphatase. *J. Biol. Chem.* **251**:2307

Garrahan, P.J., Rega, A.F., Alonso, G.L. 1976. The interaction of magnesium ions with the calcium pump of sarcoplasmic reticulum. *Biochim. Biophys. Acta* **448**:121

Ginsburg, H., Tosteson, M.T., Tosteson, D.C. 1978. Some effects of trinitrocresolate and valinomycin on Na and K transport across thin lipid bilayer membranes: A steady-state analysis

with simultaneous tracer and electrical measurements. *J. Membrane Biol.* **42**:153

Hasselbach, W., Makinose, M. 1963. Ueber den Mechanismus des Calciumtransportes durch die Membranen des sarkoplasmatischen Reticulums. *Biochem. Z.* **339**:94

Haynes, D.H., Chiu, V.C.K. 1977. Kinetics of passive Ca^{2+} transport by skeletal sarcoplasmic reticulum. *In:* Calcium-Binding Proteins and Calcium Function. R.H. Wasserman, R.A. Corradino, E. Carafoli, R.H. Kretsinger, D.H. MacLennan, and F.L. Siegel, editors pp. 137. North Holland, New York-Amsterdam-Oxford

Haynes, D.H., Chiu, V.C.K. 1978. 1-anilino-8-naphthalenesulfonate as a fluorescent probe of calcium transport: Application to skeletal sarcoplasmic reticulum. *Ann. N.Y. Acad. Sci.* **307**:217

Haynes, D.H., Pressman, B.C. 1974. Complexation by ionophores. *J. Membrane Biol.* **18**:1

Haynes, D.H., Simkowitz, P. 1977. 1-anilino-8-naphthalenesulfonate: A fluorescent probe of ionophore and ionophore transport kinetic and transmembrane asymmetry. *J. Membrane Biol.* **33**:63

Ikemoto, N. 1974. The calcium binding sites involved in the regulation of purified adenosine triphosphatase of the sarcoplasmic reticulum. *J. Biol. Chem.* **249**:649

Inesi, G., Croan, C., Verjovski-Almeida, S., Kurzmack, M., Lewis, D.E. 1978*a*. Mechanism of free energy utilization for active transport of calcium ion. *In:* Frontiers of Biological Energetics, Vol. II. L. Dutton, J. Lee, and A. Scarpa, editors pp. 1212. Academic Press, New York

Inesi, G., Kurzmack, M., Verjovski-Almeida, S. 1978*b*. ATPase phosphorylation and calcium ion translocation in the transient state of sarcoplasmic reticulum activity. *Ann. N.Y. Acad. Sci.* **307**:224

Inesi, G., Maring, E., Murphy, A.J., McFarland, B.H. 1970. A study of the phosphorylated intermediate of sarcoplasmic reticulum ATPase. *Arch. Biochem. Biophys.* **138**:285

Inesi, G., Scarpa, A. 1972. Fast kinetics of adenosine triphosphate dependent Ca^{2+} uptake by fragmented sarcoplasmic reticulum. *Biochem.* **11**:356

Kalbitzer, H.R., Stehlik, D., Hasselbach, W. 1978. The binding of calcium and magnesium to sarcoplasmic reticulum vesicles as studied by manganese electron paramagnetic resonance. *Eur. J. Biochem.* **82**:245

Kanazawa, T., Yamada, S., Yamamoto, T., Tonomura, Y. 1971. Reaction mechanism of Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. *J. Biochem.* **70**:95

MacLennan, D.H., Holland P.C. 1975. Calcium transport in sarcoplasmic reticulum. *Annu. Rev. Biophys. Eng.* **4**:377

Makinose, M., Hasselbach, W. 1965. Der Einfluß von Oxalat auf den Calcium-Transport isolierter Vesikel des sarkoplasmatischen Reticulum. *Biochem. Z.* **343**:360

McIntosh, D.B., Berman, M.C. 1978. Calcium ion stabilization of the calcium transport system of sarcoplasmic reticulum. *J. Biol. Chem.* **253**:5140

McKinley, D., Meissner, G. 1977. Sodium and potassium ion permeability of sarcoplasmic reticulum vesicles. *FEBS Lett.* **82**:47

McKinley, D., Meissner, G. 1978. Evidence for a K^+, Na^+ permeable channel in sarcoplasmic reticulum. *J. Membrane Biol.* **44**:159

Meissner, G. 1973. ATP and Ca^{2+} binding by the Ca^{2+} pump protein of sarcoplasmic reticulum. *Biochim. Biophys. Acta* **298**:906

Meissner, G. 1975. Isolation and characterization of two types of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* **389**:51

Murphy, A.J. 1978. Effects of divalent cations and nucleotides on the reactivity of the sulfhydryl groups of sarcoplasmic reticulum membranes. *J. Biol. Chem.* **253**:385

Neet, K.E., Green, N.M. 1977. Kinetics of the cooperativity of the Ca^{2+} transporting adenosine triphosphatase of sarcoplasmic reticulum and the mechanism of the ATP interaction. *Arch. Biochem. Biophys.* **178**:588

Noack, E., Kurzmack, M., Verjovski-Almeida, S., Inesi, G. 1978. The effect of propanolol and its analogues on Ca^{2+} transport by sarcoplasmic reticulum vesicles. *J. Pharm. Exp. Ther.* **206**:281

Panet, R., Pick, U., Selinger, Z. 1971. The role of calcium and magnesium in the adenosine triphosphatase reaction of sarcoplasmic reticulum. *J. Biol. Chem.* **246**:7349

Pressman, B.C., Haynes, D.H. 1969. Ionophorous agents as mobile ion carriers. *In:* Symposium on the Molecular Basis of Membrane Function. D.C. Tosteson, editor. p. 221. Prentice Hall, New York

Russell, J.T., Beeler, T., Martonosi, A. 1979*a*. Optical probe responses on sarcoplasmic reticulum: Oxacarbocyanines. *J. Biol. Chem.* **254**:2040

Russell, J.T., Beeler, T., Martonosi, A. 1979*b*. Optical probe responses on sarcoplasmic reticulum: Merocyanine and oxonol dyes. *J. Biol. Chem.* **254**:2047

Shamoo, A.E., MacLennan, D.H. 1975. Separate effects of mercurial compounds on the ionophoric and hydrolytic functions of the $(Ca^{2+}+Mg^{2+})$-ATPase of sarcoplasmic reticulum. *J. Membrane Biol.* **25**:65

Shigekawa, M., Dougherty, J.P. 1978*a*. Reaction mechanism of Ca^{2+}-dependent ATP hydrolysis by skeletal muscle sarcoplasmic reticulum in the absence of added alkali metal salts. II. Kinetic properties of the phosphoenzyme formed at the steady state in high Mg^{2+} and low Ca^{2+} concentrations. *J. Biol. Chem.* **253**:1451

Shigekawa, M., Dougherty, J.P. 1978*b*. Reaction mechanism of Ca^{2+}-dependent ATP hydrolysis by skeletal muscle sarcoplasmic reticulum in the absence of added alkali metal salts. III. Sequential occurrence of ADT-sensitive and ADP-insensitive phosphoenzymes. *J. Biol. Chem.* **253**:1458

Ueno, T., Sekine, T. 1978. Study on calcium transport by sarcoplasmic vesicles using fluorescence probes. *J. Biochem.* **84**:787

Vanderkooi, J.M., Ierokomas, A., Nakamura, H., Martonosi, A. 1977. Fluorescence energy transfer between Ca^{2+} transport ATPase molecules in artificial membrane. *Biochemistry* **16**:1262

Verjovski-Almeida, S., Inesi, G. 1979. Fast-kinetic evidence for an activating effect of ATP on the Ca^{2+} transport of sarcoplasmic reticulum ATPase. *J. Biol. Chem.* **254**:18

Vianna, A.L. 1975. Interaction of calcium and magnesium in activating and inhibiting the nucleoside triphosphatase of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta* **410**:389

Walaas, E. 1958. Stability constants of metal complexes with mononucleotides. *Acta Chim. Scand.* **12**:528

Weber, A., Herz, R., Reiss, I. 1966. Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. *Biochem. Z.* **345**:329

Yamada, S., Yamamoto, T., Tonomura, Y. 1970. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. *J. Biochem.* **67**:789

Yamamoto, T., Tonomura, Y. 1967. Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle. I. Kinetic studies. *J. Biochem.* **62**:558

Zimniak, P., Racker, E. 1978. Electrogenicity of Ca^{2+} transport catalyzed by the Ca^{2+}-ATPase from sarcoplasmic reticulum. *J. Biol. Chem.* **253**:4631

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