Biochimica et Biophysica Acta, 298 (1973) 270–278

BBA 76212

CALCIUM TRANSPORT BY RABBIT SKELETAL MUSCLE MICROSOMES ("FRAGMENTED SARCOPLASMIC RETICULUM")

ARNOLD M. KATZ* and DORIS I. REPKE

Division of Cardiology, Department of Medicine, Mount Sinai School of Medicine, City University of New York, 100th Street at 5th Avenue, New York, N. Y. 10029 (U.S.A.)

(Received October 2nd, 1972)

SUMMARY

Ca^{2+} binding by skeletal muscle microsomes in 5 mM ATP exhibited saturation kinetics in the range of Ca^{2+} concentrations between $3 \cdot 10^{-8}$ and 10^{-5} M. Approximately 140 nmoles binding sites per mg protein were found. These had a Ca^{2+} binding constant of approximately $4.5 \cdot 10^{6}$ M^{-1} with half saturation at $2.2 \cdot 10^{-7}$ M Ca^{2+}. In the presence of oxalate, much larger amounts of Ca^{2+} (approx. 6 μmoles/mg protein) were taken up by the microsomes (Ca^{2+} uptake), but the rate of Ca^{2+} uptake increased linearly with $[Ca^{2+}]$ when ionized Ca^{2+} concentrations were below $3 \cdot 10^{-6}$ M. At Ca^{2+} concentrations above $3 \cdot 10^{-6}$ M, Ca^{2+} uptake was inhibited. Double reciprocal plots of the Ca^{2+} dependence of the initial rates of Ca^{2+} uptake in the concentration range between $3 \cdot 10^{-7}$ M and 10^{-5} M, unlike those of Ca^{2+} binding, did not demonstrate saturation kinetics, but could be interpreted as representing a non-saturable system with inhibition at higher Ca^{2+} concentrations. In view of these differences, and because Ca^{2+}-binding sites were almost fully saturated at 10^{-6} M Ca^{2+}, whereas Ca^{2+} uptake rate increased linearly with increasing $[Ca^{2+}]$ to approximately $3 \cdot 10^{-6}$ M, the Ca^{2+}-binding sites are not shown kinetically to participate in oxalate-dependent Ca^{2+} uptake.

INTRODUCTION

A central role for Ca^{2+} in the excitation–contraction coupling of muscle is well established[1–6]. Ca^{2+} initiates contraction when it is bound to troponin, the Ca^{2+} receptor of the contractile proteins, whereas relaxation occurs when Ca^{2+} is removed from troponin. In skeletal muscle this latter process is effected by the sarcoplasmic reticulum, an intracellular membrane network which can remove Ca^{2+} from the high-affinity Ca^{2+}-binding site of troponin. Skeletal muscle microsomes, which contain fragmented sarcoplasmic reticulum, can remove Ca^{2+} from solution and cause relaxation of sensitive contractile protein systems *in vitro*. In the presence of Mg^{2+} and ATP, small amounts of Ca^{2+} rapidly become associated with these microsomes by a mechanism designated Ca^{2+} binding. When oxalate, or high concentrations of inorganic phosphate are included, a much greater amount of Ca^{2+}

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

* Philip J. and Harriet L. Goodhart Professor of Medicine (Cardiology).

becomes associated with the microsomes by a slower process designated Ca^{2+} uptake, and calcium oxalate or calcium phosphate precipitates can be demonstrated within the microsomal vesicles[4]. Because Ca^{2+} binding is much more rapid than Ca^{2+} uptake, the binding of Ca^{2+} to a specific transport site might represent an early step in the process that effects Ca^{2+} uptake. Alternatively Ca^{2+} binding and Ca^{2+} uptake could represent two independent mechanisms. To define the relationship between these two *in vitro* processes of Ca^{2+} sequestration, kinetic analyses of the dependence of the amount of Ca^{2+} binding and the rate of Ca^{2+} uptake upon Ca^{2+} concentration were carried out.

METHODS

Microsomes were prepared from rabbit white skeletal muscle by the methods of Harigaya and Schwartz[7] (H_2O-microsomes) and Katz *et al.*[8] (suc-microsomes). Ca^{2+} binding and Ca^{2+} uptake were measured at 25 °C by Millipore filtration[8,9] of solutions containing 5 mM MgATP, 0.12 M KCl and 40 mM histidine at pH 6.8. The level of ionized Ca^{2+} was adjusted by the use of various concentrations of ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA) in solutions containing a total $^{45}CaCl_2$ concentration of 0.025 mM (approx. 0.05 μCi/ml in the final reaction mixture). Ionized Ca^{2+} concentrations were calculated by the equations of Katz *et al.*[8]. Although there remains some disagreement as to the constants for the affinities of ATP and EGTA for Ca^{2+}, this would not influence the kinetic differences noted in this study because the same Ca^{2+}–EGTA buffers were used in both the Ca^{2+} uptake and Ca^{2+} binding studies.

Samples for Ca^{2+} binding (without oxalate) were obtained at 30-s intervals for several minutes after the $^{45}Ca^{2+}$–EGTA buffer was added. The value for the amount of Ca^{2+} bound was calculated from the maximal amount of Ca^{2+} removed from solution by the microsomes. Except at initial Ca^{2+} concentrations below $1.32 \cdot 10^{-7}$ M, binding approached a maximum within 0.5 to 2 min after addition of the Ca^{2+} buffer. At lower initial Ca^{2+} concentrations, this maximum was approximated at 2–4 min. A much slower increase in apparent Ca^{2+} binding was occasionally seen after the initial rapid phase of binding was completed, due possibly to Ca^{2+} uptake in the presence of P_i liberated from ATP. With other preparations, a slight decrease in Ca^{2+} was seen, however, 2–5 min after addition of the $^{45}Ca^{2+}$–EGTA buffer[10]. This "release" phenomenon, when it was seen, occurred sooner at higher Ca^{2+} concentrations. After repeated studies of these phenomena, it was found that a 4-min sample time gave reasonably accurate values for the peak of the initial rapid phase of Ca^{2+} binding. The maximal error resulting from these poorly understood, but well documented, phenomena were no more than 10% at the higher Ca^{2+} concentrations ($> 10^{-6}$ M), being much less for almost all other measurements. In all experiments, the amount of Ca^{2+} binding was related to the final ionized Ca^{2+} concentration in the solution, appropriate corrections being made to account for the Ca^{2+} associated with the microsomes.

Calcium uptake was measured with 5–10 μg microsomal protein per ml in solutions containing 2.5 mM Tris–oxalate. Ca^{2+} uptake rates were calculated from a series of samples filtered at appropriate times after addition of the $^{45}Ca^{2+}$–EGTA buffers. Accurate determinations of Ca^{2+} uptake rates required measurements at

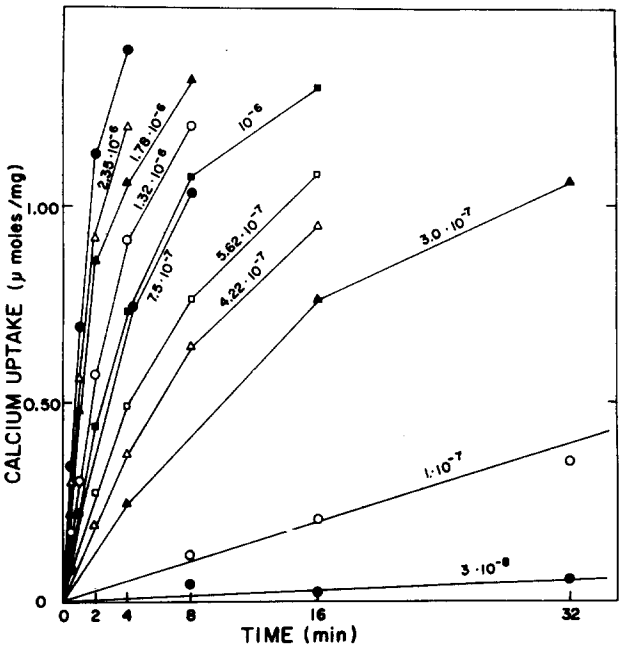

Fig. 1. Time dependence of Ca^{2+} uptake of suc-microsomes at Ca^{2+} concentrations of $3 \cdot 10^{-8}$ M (●), 10^{-7} M (○), $3 \cdot 10^{-7}$ M (▲), $4.22 \cdot 10^{-7}$ M (△), $5.62 \cdot 10^{-7}$ M (□), $7.5 \cdot 10^{-7}$ M (●), 10^{-6} M (■), $1.32 \cdot 10^{-6}$ M (○), $1.78 \cdot 10^{-6}$ M (▲), $2.35 \cdot 10^{-6}$ M (△), and $3 \cdot 10^{-6}$ M (●). The rate of Ca^{2+} uptake was measured from the initial slope of these curves.

numerous time intervals after the reactions were initiated (Fig. 1). If only a single sample time was used over this range of $[Ca^{2+}]$, misleading results demonstrating an apparent, but not real, fall in the rate of Ca^{2+} uptake at the higher Ca^{2+} concentrations would be obtained because of the widely differing rates of Ca^{2+} uptake. Uptake rates were calculated directly from the initial slopes of these uptake curves. Alternatively, rates could be determined from plots of log $C(t)/C(o)$ against t, when $C(t)=Ca^{2+}$ concentration at time t, $C(o)=Ca^{2+}$ concentration at zero time. This latter method gave results similar to those obtained by determining the initial tangents to curves such as shown in Fig. 1. The pH was maintained at 6.8 during the course of these experiments by the 40 mM histidine buffer. The possibility that the slowness with which the Ca^{2+}–EGTA complex dissociates could influence the uptake rates determined in this study can be excluded because the rate of Ca^{2+} release from Ca^{2+}–EGTA[11] is approximately 100 times faster than measured Ca^{2+} uptake.

Radioactivity was counted in a Packard Tri-Carb Scintillation Counter. The use of 50-μl samples added to 15 ml Bray's solution prevented significant quenching or precipitation of Ca^{2+} during counting. Lines were computed for kinetic plots by a PDP-8e computer, using the method of least squares.

RESULTS

Initial studies, carried out with microsomes prepared in sucrose by the method of Katz *et al.*[8] indicated that at Ca^{2+} concentrations between 10^{-7} and $3 \cdot 10^{-6}$ M,

Ca^{2+} binding demonstrated saturation kinetics, whereas the rate of Ca^{2+} uptake increased linearly with increasing [Ca^{2+}] (Fig. 2). The data illustrated in Fig. 2 were examined by two systems of graphic analysis, and lines were drawn by the method of least squares with the assumption that the points defined a single straight line. Double-reciprocal plots indicated the presence of approximately 80 nmoles of Ca^{2+} binding sites per mg protein having a Ca^{2+} binding constant of approximately $6 \cdot 10^6$ M^{-1} (Fig. 3). When Ca^{2+} uptake was examined in this manner the extrapolated line went through the origin (Fig. 3), again indicating the absence of a saturable system of Ca^{2+}-transport sites. Scatchard plots of these data also demonstrated saturation kinetics for Ca^{2+} binding, the data in this range of Ca^{2+} concentration approximating a straight line, indicating the existence of a single class of high affinity Ca^{2+} binding sites. In the case of Ca^{2+} uptake, the ratio: uptake

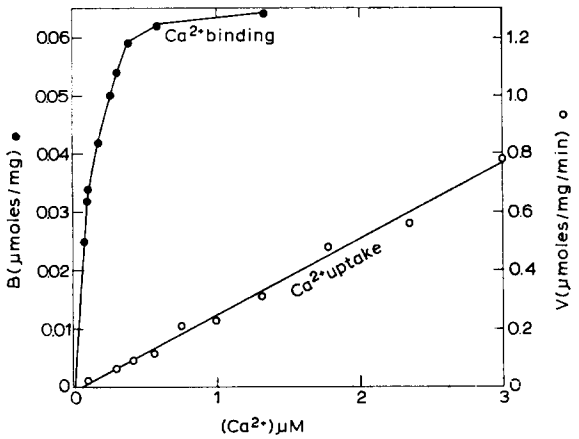

Fig. 2. Direct plot of Ca^{2+} dependence of Ca^{2+} binding, B, (●) and the rate of Ca^{2+} uptake, V, (○) of suc-microsomes. Experimental methods as described in text. Ca^{2+} binding was measured with 0.2 mg/ml protein, whereas Ca^{2+} uptake was measured with 0.005 mg/ml protein. The same Ca^{2+} buffers were used in both studies.

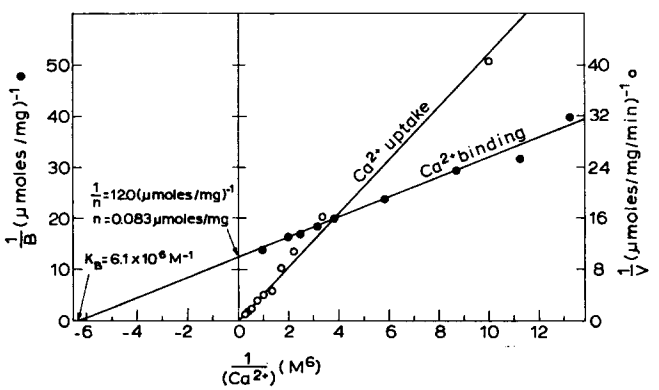

Fig. 3. Double reciprocal plots of Ca^{2+} binding (●) and Ca^{2+} uptake (○) of suc-microsomes based on data in Fig. 2. In this experiment, the value for n (number of binding sites per mg protein), was 83 nmoles/mg and K_B, (the affinity constant for Ca^{2+}) was $6.1 \cdot 10^6$ M^{-1}.

rate/free Ca^{2+} concentration was essentially independent of the rate of uptake (Fig. 4).

Because both the maximum Ca^{2+} uptake and the number of Ca^{2+}-binding sites of the suc-microsomes were less than those reported by others, these studies were repeated with H_2O-microsomes which were prepared in dilute bicarbonate buffer and washed with KCl[7]. Kinetic studies of these microsomes, which showed maximal Ca^{2+} uptake of approximately 6 μmoles/mg, were entirely similar to those

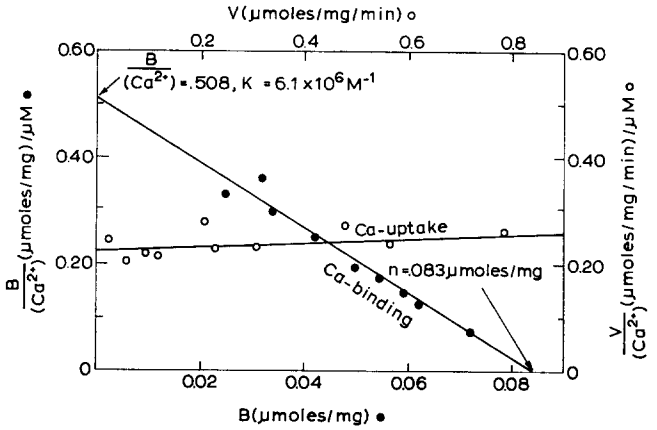

Fig. 4. Scatchard plots of Ca^{2+} binding (●) and Ca^{2+} uptake (○) based on data in Fig. 2. The values for n and K_B are the same as those obtained from the double reciprocal plot (Fig. 3).

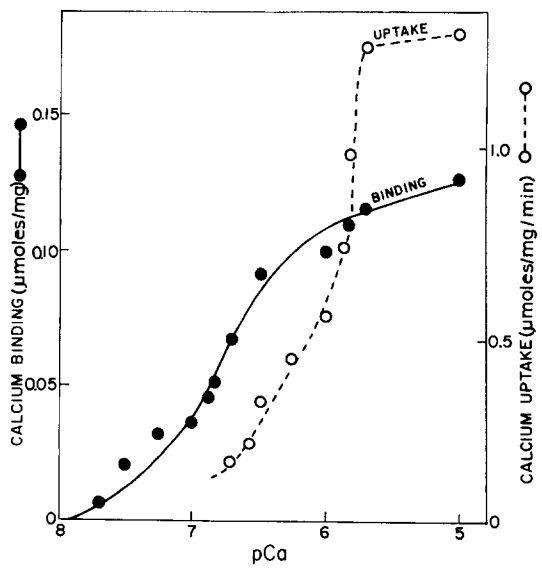

Fig. 5. Semilogarithmic plot of Ca^{2+} uptake and Ca^{2+} binding by H_2O-microsomes. Ca^{2+} uptake was measured with 0.0075 mg/ml protein at Ca^{2+} concentrations between 10^{-7} and $4.22 \cdot 10^{-7}$. A Ca^{2+} concentration $> 4.22 \cdot 10^{-7}$ M, 0.005 mg/ml protein was used. Ca^{2+} binding was measured with 0.1 mg/ml protein as described in Methods, samples being taken 4 min after the reaction was started.

described above. The Ca^{2+} dependence of Ca^{2+}-uptake rate was significantly different from that for Ca^{2+} binding (Fig. 5), demonstrating, as in Fig. 2, that Ca^{2+} uptake increased significantly at Ca^{2+} concentrations where Ca^{2+} binding was already 90% saturated. Ca^{2+} binding of the H_2O-microsomes, like that of suc-microsomes, exhibited saturation kinetics (Fig. 6) with approximately 140 nmoles binding sites per mg protein and an affinity constant of approximately $4.5 \cdot 10^6$ M^{-1}. As was found for suc-microsomes, double reciprocal plots of the Ca^{2+} dependence of Ca^{2+} uptake rate did not give positive values for $1/K_m$ when lines were drawn using points obtained at Ca^{2+} concentrations below 10^{-5} M (Fig. 7). Extrapolations in a number of experiments crossed the origin at positive values for $1/K_m$ as often as they did at negative values. The curvature of the actual line from the extrapolated line at the higher Ca^{2+} concentrations reflects inhibition of Ca^{2+}-uptake rate (see Discussion). The

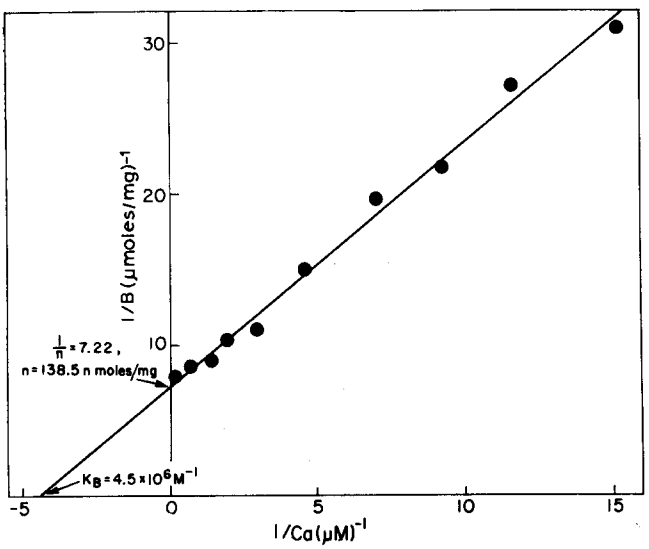

Fig. 6. Double reciprocal plot of Ca^{2+} binding by H_2O-microsomes.

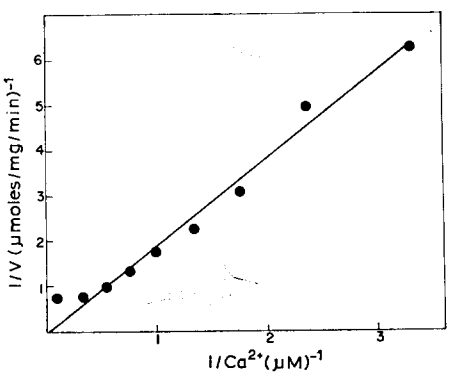

Fig. 7. Double reciprocal plot of Ca^{2+} uptake of H_2O-microsomes. The line was drawn by the method of least mean squares using data obtained at Ca^{2+} concentrations $< 3 \cdot 10^{-6}$ M.

maximal rate of Ca^{2+} uptake, like the total Ca^{2+} uptake, was approximately twice as high for these microsomes when compared to that for microsomes prepared in sucrose.

DISCUSSION

A number of investigators have examined Ca^{2+} binding and Ca^{2+} uptake by skeletal microsomes, but there are few kinetic studies of these processes. Kinetic data have generally been presented as semilogarithmic plots, in which the amount of Ca^{2+} bound or the rate of Ca^{2+} uptake is plotted against the negative logarithm of Ca^{2+} concentration[4,5,12]. In the case of Ca^{2+} uptake, however, such plots are difficult to interpret, because of well documented inhibitory effects of higher Ca^{2+} concentrations[4,5,9]. Furthermore, the inference of kinetic interpretations from the semilogarithmic plot is subject to error[13].

The present value of $4.5 \cdot 10^6$ M^{-1} for the affinity constant of Ca^{2+} binding by microsomes prepared by the method of Harigaya and Schwartz[7] corresponds to a Ca^{2+} concentration at half saturation of $2.2 \cdot 10^{-7}$ M. This agrees well with previously reported values of K_B[5,12,14], as does the finding that Ca^{2+} binding follows saturation kinetics.

The observation that the Ca^{2+} dependence of Ca^{2+} uptake follows different kinetics than Ca^{2+} binding has not been emphasized previously. However, when the Ca^{2+} uptake rates at low Ca^{2+} concentrations reported by Hasselbach[4] and Weber *et al.*[5] are replotted as double reciprocal and Scatchard plots, interpretations similar to those documented in this report are obtained. Although these studies include fewer points than in the present report, double reciprocal plots of the published data defining the Ca^{2+} dependence of Ca^{2+} uptake rate tend to go through the origin, and the ratio: uptake rate/$[Ca^{2+}]$ does not show a clear decrease as uptake rate increased. Quantitative comparisons of these data with those in the present report is difficult, however, because different values for the Ca^{2+}–ATP, Mg^{2+}–ATP and Ca^{2+}–EGTA binding constants have been used. The data given in Fig. 5 of the present report are quite similar to those in Fig. 16 of the paper by Weber *et al.*[5], whereas Fig. 15 of the paper by Hasselbach[4] (in which uptake rates are related to mg N rather than mg protein) indicates a shift of the present curve to the left by about 0.5 pCa. This apparent shift, which can be explained by differences in selection of values for the above mentioned binding constants, does not, however, invalidate the general similarities between kinetic analyses of both the present and previously reported data. A low value for $1/K_m$ of Ca^{2+} uptake was published more recently by Worsfold and Peter[15], who found double reciprocal plots to cross the abcissa at slightly negative values for $1/Ca^{2+}$. Because the inhibitory effect of high Ca^{2+}, noted here as well as by others, causes an upturn of the line drawn in the double reciprocal plot as it approaches the origin, inclusion of such data might give the misleading impression that $-1/Ca^{2+}$ extrapolates to a high value when $1/V=0$. If the data at Ca^{2+} concentrations greater than 10^{-5} M in this latter study are excluded, however, double reciprocal plots, like those for Ca^{2+} uptake in Figs 3 and 7, cross at, or very near, the origin.

The present findings do not exclude the possibility that skeletal microsomal Ca^{2+} uptake is a saturable process, either with a very high K_m or with complex

kinetic characteristics, due for example to interacting transport sites. The latter possibility is consistent with the inhibitory effect of high Ca^{2+} concentrations, which clearly demonstrates that these Ca^{2+}-membrane interactions include more than one type of effect. This inhibitory action of Ca^{2+}, which has been noted by others[4,5,9], precludes identification of a saturable process with a high K_m ($>10^{-5}$ M). A K_m for Ca^{2+} uptake in the range approximating that for Ca^{2+} binding, approximately $2 \cdot 10^{-7}$ M, can, however, be excluded with a reasonable degree of certainty by these rate data, thus indicating that Ca^{2+} uptake and Ca^{2+} binding follow different kinetics. Furthermore, it is apparent that the Ca^{2+} binding sites become saturated at Ca^{2+} concentrations that are much lower than those at which Ca^{2+} uptake becomes inhibited (Figs 2 and 5). The kinetic data for Ca^{2+} uptake reported here are similar to those reported by Carafoli *et al.*[16] for blowfly mitochondrial Ca^{2+} uptake, which also appears not to exhibit saturation kinetics. The microsomal Ca^{2+} uptake which we have characterized cannot be attributed to the activity of mitochondrial fragments, however, because the microsomal Ca^{2+} uptake measured in these studies is unaffected by oligomycin (4 μg/mg protein) and is inhibited by only about 10% in the presence of 10 mM dinitrophenol. Furthermore mammalian mitochondria, unlike those of the blowfly, appear to take up Ca^{2+} by a saturable process that does not utilize oxalate[17].

The present findings indicate that microsomal Ca^{2+} binding is effected by a limited number of high-affinity sites, whereas Ca^{2+} uptake appears to represent a process that does not involve a system of saturable high affinity Ca^{2+}-transport sites, a mechanism already proposed to account for the translocation of Ca^{2+} by certain mitochondria. In the latter, it has been proposed that an electrochemical gradient generated by electron transport causes Ca^{2+} to enter mitochondria by means of a simple non-facilitated diffusion process[16]. In the case of skeletal microsomes, an analogous electrochemical gradient might be generated by ATP hydrolysis, although the exact relationship between Ca^{2+} uptake and ATPase activity remains in dispute[4,6,18]. The possibility exists that rapid complexing of Ca^{2+} with oxalate prevents saturation of Ca^{2+} binding sites in the sequence: Ca^{2+} binding+translocation$\rightarrow Ca^{2+}$ release within the vesicles$\rightarrow$ complexing of Ca^{2+} with oxalate. This explanation for the lack of saturation kinetics for Ca^{2+} uptake assumes that the rate of Ca^{2+} release and complexing with oxalate is so great as to prevent saturation of the Ca^{2+}-binding sites. While such an explanation seems unlikely as the overall rate of Ca^{2+} uptake is much slower than that of Ca^{2+} binding, additional data on the reaction mechanism for Ca^{2+} uptake will be needed to define more precisely the relationship between Ca^{2+} binding and Ca^{2+} uptake. It has recently been shown that treatment with oleic acid or lysolecithin abolishes the ability of microsomes to accumulate Ca^{2+} (Ca^{2+} uptake) without altering either ATPase activity or the number and affinity of the Ca^{2+}-binding sites[19]. These observations agree well with the present findings that demonstrate that Ca^{2+} binding and Ca^{2+} uptake represent two kinetically different processes, as do similar studies with cardiac microsomes[20].

ACKNOWLEDGEMENTS

This work was supported by Research Grants HL-13191 from the U.S. Public Health Service and a Grant-in-Aid from the New York Heart Association.

REFERENCES

1 Sandow, A. (1965) *Pharmacol. Rev.* 17, 265
2 Weber, A., Herz, R. and Reiss, I. (1963) *J. Gen. Physiol.* 46, 679
3 Katz, A. M. (1970) *Physiol. Rev.* 50, 63
4 Hasselbach, W. (1964) *Prog. Biophys.* 14, 167
5 Weber, A., Herz, R. and Reiss, I. (1966) *Biochem. Z.*, 345, 329
6 Ebashi, S. and Endo, M. (1968) *Prog. Biophys. Mol. Biol.* 18, 123
7 Harigaya, S. and Schwartz, A. (1969) *Circ. Res.* 25, 781
8 Katz, A. M., Repke, D. I., Upshaw, J. E. and Polascik, M. A. (1970) *Biochim. Biophys. Acta* 205, 473
9 Martonosi, A. and Feretos, R. (1964) *J. Biol. Chem.* 239, 648
10 Entman, M. L., Bornet, E. P. and Schwartz, A. (1972) *J. Mol. Cell. Cardiol.* 4, 155
11 Hellam, D. C. and Podolsky, R. J. (1969) *J. Physiol. London* 200, 807
12 Carvalho, A. P. and Leo, B. (1967) *J. Gen. Physiol.* 50, 1327
13 Edsall, J. T. and Wyman, J. (1958) *Biophysical Chemistry*, Chapter 11, Academic Press, New York
14 Ogawa, Y. (1970) *J. Biochem. Tokyo* 67, 667
15 Worsfold, M. and Peter, J. B. (1970) *J. Biol. Chem.* 245, 5545
16 Carafoli, E., Hansford, R. G., Sacktor, B. and Lehninger, A. L. (1971) *J. Biol. Chem.* 246, 964
17 Carafoli, E. and Lehninger, A. (1971) *Biochem. J.* 122, 681
18 Seraydarian, K. and Mommaerts, W. F. H. M. (1965) *J. Cell Biol.* 26, 641
19 Fiehn, W. and Migala, A. (1971) *Eur. J. Biochem.* 20, 245
20 Repke, D. I. and Katz, A. M. (1972) *J. Mol. Cell. Cardiol.* 4, 401