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Calcium Binding to the Sarcoplasmic Reticulum of Rabbit Skeletal Muscle*

Jean Chevallier† and Ronald A. Butow‡

ABSTRACT: Passive calcium binding to the sarcoplasmic reticulum of rabbit skeletal muscle has been studied. Previous results obtained by Carvalho, and Cohen and Selinger are reevaluated in view of artifacts which may arise due to endogenous, exchangeable calcium in the preparations. By washing or treating membrane preparations with Chelex-100, and by carrying out binding assays by equilibrium dialysis against a relatively large amount of free calcium, we can distinguish three types of calcium binding sites on the membranes. About 80–90% is nonspecific and display a dissociation constant for calcium of about 0.32 mM. The two remaining sites are designated as calcium specific since the calcium binding to these sites persists in the presence of 0.6 M KCl and 1 mM $MgCl_2$.

In the absence of KCl and $MgCl_2$, the sites of highest affinity bind about 10–20 nmoles of calcium/mg of protein and display a dissociation constant of 0.4 μM; the sites of lower affinity bind about 130 nmoles of calcium/mg of protein with a dissociation constant of 13 μM. In the presence of KCl and $MgCl_2$, the dissociation constants for both the high- and the low-affinity sites increase about threefold. About 10 and 90 nmoles of calcium are bound per mg of protein to the high- and low-affinity sites, respectively. The lanthanide ions La^{3+}, Gd^{3+}, and Yb^{3+}, which are known to display a very high affinity toward anionic sites because of their high charge density, are nevertheless relatively poor inhibitors of calcium binding to the specific sites, illustrating the high degree of specificity for calcium at these sites. La^{3+} inhibition of calcium binding at the calcium specific sites is of the competitive type with a K_i of 6.5 μM.

The relaxation process in skeletal muscle involves the removal of calcium from myofibrils by the sarcoplasmic reticulum (Weber *et al.*, 1963; Weber, 1966). Isolated preparation of these membranes provides a simple system for studying ion transport, since their major function is the active translocation of calcium ions (Martonosi and Feretos, 1964). In the presence of ATP, magnesium, and a "trapping" agent such as oxalate, these vesicular preparations can transport large quantities of calcium into their internal space against a large concentration gradient (Hasselbach and Makinose, 1961; Hasselbach and Makinose, 1963; Weber, 1966).

Present concepts of the mechanism of active transport require that a preliminary and essential step for calcium translocation is the binding of the ion to the membrane. A knowledge of the number of calcium binding sites, their specificity, and relative affinities, is essential to an understanding of this process and its relationship to active transport. Aspects of this problem have been investigated by Carvalho (1966) and more recently by Cohen and Selinger (1969). In this report we present results on calcium binding which differ in part from the results obtained by these investigators. In addition, our studies have been extended to include the effects of some lanthanides on passive calcium binding.

Materials and Methods

Materials. Chelex-100 (100–200 mesh) was obtained from Bio-Rad Laboratories. ^{45}Ca was purchased from New England Nuclear as $CaCl_2$ in 0.5 N HCl (6.15 mCi/mg of Ca). Lanthanum chloride ($LaCl_3 \cdot 6H_2O$) was obtained from Fischer. Gadolinium sulfate ($Gd_2(SO_4)_3 \cdot 8H_2O$) and ytterbium sulfate ($Yb_2(SO_4)_3 \cdot 8H_2O$) were purchased from Alfa Inorganics. All other reagents were biochemical grade.

Preparation of Sarcoplasmic Reticulum. Membranes of the sarcoplasmic reticulum were prepared according to the procedure of Martonosi *et al.* (1968). After isolation and purification the preparation was stored frozen at −70° in 0.15 M KCl, 0.01 M histidine-Cl (pH 7.4), and 0.5 M sucrose at a protein concentration of 20–25 mg/ml. Prior to the binding experiments, suspensions of sarcoplasmic reticulum were prepared following two different procedures. (1) Membranes were washed two times by centrifugation with 0.01 M histidine-Cl (pH 6.5) containing 10^{-3} M $MgCl_2$ and 0.6 M KCl (buffer A).

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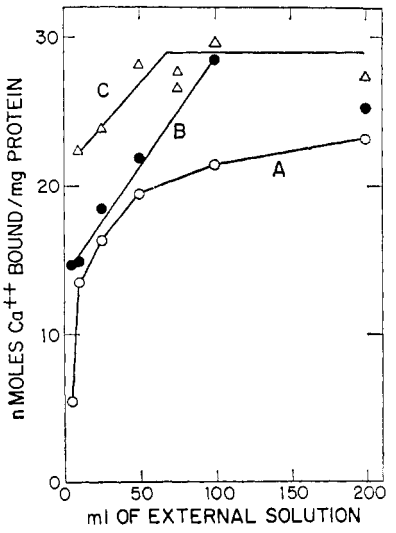

FIGURE 1: Dependence of apparent Ca^{2+} bound by the sarcoplasmic reticulum as a function of the volume of external dialyzing solution. Effect of washing and Chelex-100 treatment. Vesicles were dialyzed against different volumes of buffer A containing 10^{-5} M [^{45}Ca]$CaCl_2$ as indicated in Materials and Methods. Curve A, nontreated membranes, 0.750 mg of protein; curve B, membranes washed twice with buffer A, 0.730 mg of protein; curve C, membranes eluted through Chelex-100 column with buffer B, 0.750 mg of protein.

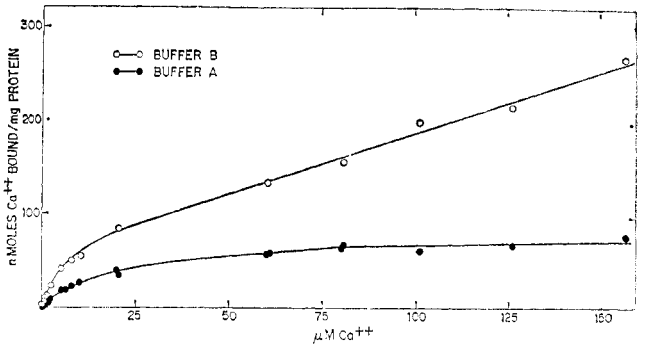

FIGURE 2: Dependence of Ca^{2+} bound by the sarcoplasmic reticulum as a function of the free Ca^{2+} concentration. Effect of KCl and $MgCl_2$. Membrane preparations were eluted through a Chelex-100 column with buffer B as described in the text. Dialysis was carried out against: 100 ml of buffer A (0.01 M histidine-Cl (pH 6.5), 10^{-3} M $MgCl_2$, and 0.6 M KCl), and 0.435 mg of protein 100 ml of buffer B (0.01 M histidine-Cl, pH 6.5) and 1.05 mg of protein (O).

The final pellet was resuspended in buffer A to a concentration of about 2 mg/ml. (2) Preparations between 20 and 50 mg of protein were applied directly on a Chelex-100 column (1 × 24 cm; Na form) equilibrated with 0.01 M histidine-Cl (pH 6.5) (buffer B). The membranes were passed through the column at a flow rate of 120 ml/hr. The breakthrough material was pooled and used directly for binding measurements. All operations were carried out at 4°.

Equilibrium Dialysis. A 0.250-ml aliquot of a suspension of the membrane preparation in either buffer A or B was placed in small 0.25-in. diameter dialysis bag (Arthur H. Thomas Co.) and dialyzed at 4° with shaking against specified volumes (see text) of buffer A or B containing various concentrations of [^{45}Ca]$CaCl_2$ (specific activity between 5×10^3 and 5×10^5 cpm per μmole). After 16 hr, 50-μl aliquots were removed from the dialysis bag and the external solution and counted by liquid scintillation in Bray's solution (Bray, 1960). In order to establish that equilibrium was reached, all dialysis experiments were carried out with a buffer control contained in a separate bag.

Determination of Endogenous Calcium. The amount of endogenous Ca^{2+} present in preparations of sarcoplasmic reticulum was determinated by atomic absorption spectroscopy. Aliquots of the sarcoplasmic reticulum were washed three times with 10 ml of 1 N $HClO_4$. $LaCl_3$ was added to the supernatant solutions to a final concentration of 1%. The amount of Ca^{2+} present in the supernatant was determined with a Perkin-Elmer atomic absorption spectrometer.

Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951).

Results

Assay of Calcium Binding. In a previous study by Cohen and Selinger (1969), the Ca^{2+} binding capacity of isolated membranes of the sarcoplasmic reticulum was determined under conditions whereby relatively high concentrations of the membranes (2–6 mg of protein) were equilibrated with 10 ml of various concentrations of [^{45}Ca]$CaCl_2$. Under these conditions, if a significant amount of endogenous Ca^{2+} was present in their membrane preparations and was exchangeable with the added [^{45}Ca]$CaCl_2$, an underestimation of the amount of Ca^{2+} bound would occur as a result of a dilution of the isotope. Analyses of the amount of endogenous Ca^{2+} in our crude preparations of the sarcoplasmic reticulum reveal that as much as 500 nmoles of Ca^{2+}/mg of protein may be present.[1] It should be noted that the method we have used for the preparation of these membranes (Martonosi *et al.*, 1968) is the same as that employed by Cohen and Selinger (1969).

It would be expected that endogenous Ca^{2+}, exchangeable with the external [^{45}Ca]$CaCl_2$ solution, would have the effect of decreasing the amount of apparent Ca^{2+} bound with decreasing volumes of the external solution. The data presented in Figure 1 are clearly consistent with this view. Using the equilibrium dialysis procedure described in Materials and Methods, the amount of apparent Ca^{2+} bound by untreated membranes is seen to decrease dramatically as the volume of the external solution is decreased (curve A, Figure 1). However, if the membrane preparations are washed two times with buffer A (curve B, Figure 1) or eluted through a Chelex-100 column (curve C, Figure 1) in order to facilitate removal of endogenous Ca^{2+}, the apparent binding is then greater at smaller volumes of the external dialyzing medium compared to the untreated preparations. We have determined that these procedures remove up to 50% of the endogenous Ca^{2+}. With Chelex-100-treated preparations, no significant change in apparent Ca^{2+} binding occurs from 50 to 200 ml of external solution. Thus, in order to minimize errors in the estimation of Ca^{2+} binding due to exchange by endogenous Ca^{2+}, we have routinely carried out binding measurements with either washed or Chelex-100-treated preparations with at least 100 ml of external dialysis solution.

From these results, we were prompted to reinvestigate the passive Ca^{2+} binding properties of the sarcoplasmic reticulum. Our assay conditions should first of all, reduce the possibilities of errors in estimation of binding parameters, and secondly,

[1] It should be pointed out that this value is about 20 times greater than that reported by Makinose and Hasselbach (1965) and by Duggan and Martonosi (1970). The reason for this discrepancy is not clear at the present time.

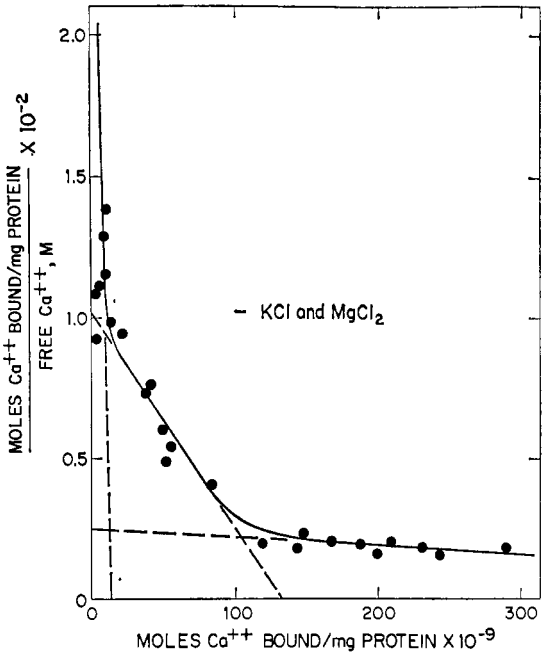

FIGURE 3: Scatchard plot for the binding of Ca^{2+} by sarcoplasmic reticulum vesicles. Binding assays were carried out with 1.05 mg of membrane protein eluted through a Chelex-100 column; dialysis was carried out against 100 ml of buffer B. The free Ca^{2+} concentration was varied between 2.7×10^{-7} and 1.5×10^{-4} M.

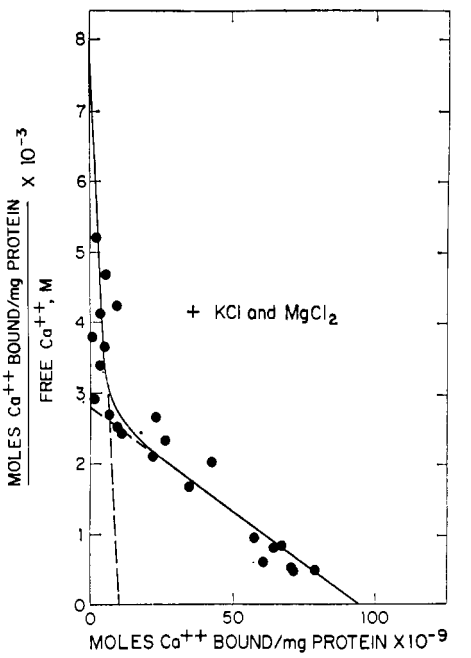

FIGURE 4: Scatchard plot for the binding of Ca^{2+} by sarcoplasmic reticulum vesicles. Binding assays were carried out with 0.435 mg of membrane protein eluted through a Chelex-100 column; dialysis was carried out against 100 ml of buffer A. The free Ca^{2+} concentration was varied between 2.9×10^{-7} and 1.5×10^{-4} M.

permit detection of a small number of high affinity Ca^{2+} binding sites which might otherwise be obscured if assays were carried out at relatively small ratios of equilibrating solution to membrane protein.

Passive Calcium Binding Properties of the Sarcoplasmic Reticulum. It has been demonstrated previously that cations such as K^+ and Mg^{2+} inhibit the bulk of Ca^{2+} binding to the membranes (Carvalho, 1966; Cohen and Selinger, 1969), suggesting the existence of specific and nonspecific Ca^{2+} binding sites. We have confirmed these observations using our procedure to measure Ca^{2+} binding. As shown in Figure 2, the membranes have a large capacity to bind Ca^{2+} in the absence of KCl and $MgCl_2$; saturation is not achieved at 1.5×10^{-4} $CaCl_2$. On the other hand, in the presence of 0.6 M KCl and 1 mM $MgCl_2$, Ca^{2+} saturation approaches a value of 100 nmoles of Ca^{2+}/mg of protein, which is about ten times greater than that reported by Cohen and Selinger (1969) for their Ca^{2+}-specific sites.

A further analysis of Ca^{2+} binding to the membranes is shown by Scatchard plots (Scatchard, 1949) in Figures 3 and 4. In the absence of KCl and $MgCl_2$ (Figure 3), at least three types of Ca^{2+} binding sites can be distinguished. The sites of lowest affinity, bind about 850 nmoles of Ca^{2+}/mg of protein, display a dissociation constant ($K_{dissocn}$) of 0.32 mM and probably represent nonspecific binding sites since they are eliminated by KCl and $MgCl_2$ (Figure 4). Two classes of binding sites are distinguished in the presence of $MgCl_2$ and KCl and are designated as Ca^{2+} specific. These can be further differentiated into high- and low-affinity sites. The former bind about 10 nmoles of Ca^{2+}/mg of protein with a $K_{dissocn} = 1.3$ μM and the latter, approximately 90 nmoles of Ca^{2+}/mg of protein with a $K_{dissocn} = 32$ μM (Figure 4). Thus only about 12% of the total Ca^{2+} binding capacity of the sarcoplasmic reticulum is Ca^{2+} specific. These results are summarized in Table I.

The quantitative differences between the binding parameters for the high- and the low-affinity calcium-specific sites are probably the result of the large ionic strength difference between buffer A and B.

Effect of Lanthanides on Calcium Binding. Figure 5 shows the results of comparative experiments on inhibition of Ca^{2+} binding by the trivalent lanthanide cations La^{3+}, Gd^{3+}, and Yb^{3+}. These data illustrate the high degree of specificity of the Mg^{2+}- and K^+-insensitive Ca^{2+} binding sites. At a Ca^{2+} concentration of 1 μM these cations display a similar and relatively weak affinity for the Ca^{2+}-specific high-affinity sites. Complete inhibition of Ca^{2+} binding is not achieved until the lanthanide concentration approaches 1 mM. This fact is especially striking in view of the high charge density of the lanthanides and their strong affinity for anionic sites (Lettvin *et al.*, 1964).

La^{3+} inhibition of Ca^{2+} binding to the Ca^{2+}-specific sites can be examined by the graphical procedure of Dixon (1953). Figure 6 shows a plot of the reciprocal of the amount of Ca^{2+} bound per milligram of protein against La^{3+} concentration. For three different concentrations of Ca^{2+} varied over a 25-fold range, the lines are linear and intersect, suggesting that

TABLE I: Comparison of Calcium Binding Sites.

Assay Conditions	High Affinity		Low Affinity		Nonspecific	
	n[a]	$K_{dissocn}$[b] (μM)	n	$K_{dissocn}$ (μM)	n	$K_{dissocn}$ (mM)
Buffer B	10–20	0.4	130	13	850	0.32
Buffer A	10	1.3	90	32		

[a] Amount of Ca^{2+} bound (nanomoles per milligram of protein) evaluated directly from Scatchard plots. [b] Evaluated directly from Scatchard plots.

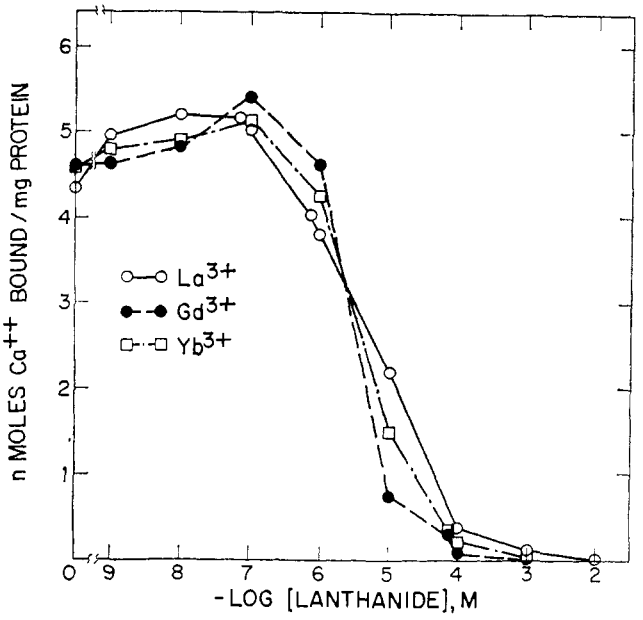

FIGURE 5: Effect of lanthanides on Ca^{2+} binding. Membrane preparations were eluted through a Chelex-100 column with buffer B prior to the assay for Ca^{2+} binding. Equilibrium dialysis experiments were carried out against 100 ml of buffer A containing 10^{-6} M [^{45}Ca]$CaCl_2$ as described in Methods and Materials. Experiments with La^{3+} represent the average values from two separate experiments with 0.525 and 0.750 mg of protein, respectively; Gd^{3+}, 0.550 mg of protein; Yb^{3+}, 0.440 mg of protein. Lanthanide solutions were prepared in buffer A just before using.

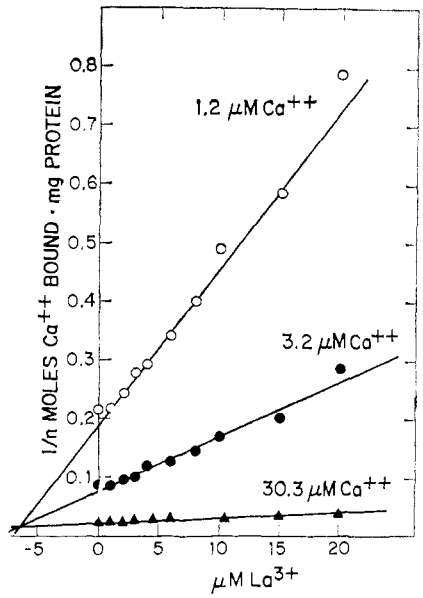

FIGURE 6: Dixon plot of La^{3+} inhibition of Ca^{2+} binding. Membrane preparations were eluted through a Chelex-100 column with buffer B as described in Materials and Methods. Dialysis was carried out with 1.1 mg of membrane protein against 100 ml of buffer A containing [^{45}Ca]$CaCl_2$ and $LaCl_3$ at the concentrations indicated in the figure. $LaCl_3$ solutions were prepared in buffer A just before using.

the K_i for La^{3+} (6.5 μM) is the same for both the high- and low-affinity Ca^{2+} binding sites. In addition, these data show that the La^{3+} inhibition is of the competitive type.

Discussion

Three types of Ca^{2+} binding sites can be distinguished on membranes of the sarcoplasmic reticulum. The majority of sites show relatively weak affinity for Ca^{2+} ($K_{dissocn}$ = 0.32 mM); their sensitivity to KCl and $MgCl_2$ indicate that they are nonspecific and most likely reflect Ca^{2+} binding to anionic phospholipid residues. In addition to these nonspecific binding sites we have demonstrated two additional Ca^{2+}-specific binding sites which differ in affinity toward Ca^{2+} and in the amount of Ca^{2+} bound by each. These results are at variance with those of Cohen and Selinger (1969) since they reported only one type of Ca^{2+}-specific site with a $K_{dissocn}$ = 40 μM. The most likely explanation for this discrepancy is the difference in the binding assay for Ca^{2+}. In particular, careful consideration must be given to the possibility of artifacts arising from endogenous Ca^{2+}. Even with a level of endogenous, exchangeable Ca^{2+} as low as 20 nmoles/g of protein (Makinose and Hasselbach (1965), Duggan and Martonosi (1970)), high-affinity binding sites ($K_{dissocn} < 5 \times 10^{-6}$ M) would tend to be obscured if binding assays are carried out at relatively high protein concentrations and low amounts of total free calcium in the medium. We have shown that these difficulties can be largely overcome by reducing the level of endogenous Ca^{2+} in the preparation, either by washing the membranes or treating them with a chelating resin, and most importantly by carrying our binding assays by equilibrium dialysis against large amounts of free Ca^{2+}. With these precautions we have determined that in the presence of KCl and $MgCl_2$ approximately 10 nmoles of Ca^{2+}/mg of protein can be bound to the high-affinity sites with a $K_{dissocn}$ = 1.3 μM and close to 100 nmoles of Ca^{2+}/mg of protein can be bound to the low-affinity sites with a $K_{dissocn}$ = 32 μM. Taken together, these Ca^{2+}-specific sites represent about 12% of the total Ca^{2+} binding capacity of the membrane.

Of considerable interest is the relationship of these three classes of Ca^{2+} binding sites to active Ca^{2+} transport. Because KCl concentrations up to 0.6 M have little effect on ATP-dependent Ca^{2+} transport (Martonosi and Feretos, 1964), the nonspecific Ca^{2+} binding sites probably do not play a role in this process. Our observation of a high-affinity Ca^{2+} binding site is consistent with the known physiological efficiency of the sarcotubular system, *i.e.*, its ability to lower the free Ca^{2+} concentration in the sarcoplasm to less than 1 μM. Recent studies by Worsfold and Peter (1970) have demonstrated the Michaelis–Menten behavior of Ca^{2+} transport in isolated sarcoplasmic reticulum vesicles. Using conditions more closely approximating physiological ones, where transport is not influenced by the oxalate trap or the total Ca^{2+} accumulating capacity of the vesicles, K_m values for Ca^{2+} of 3.0 and 12 μM were obtained with human and rat sarcotubular vesicles, respectively. Again, these values are consistent with the Ca^{2+} binding data presented here and are difficult to reconcile with a single Ca^{2+} binding site with a Ca^{2+} dissociation constant of 40 μM as reported by Cohen and Selinger (1969).

At this point it would be premature to assign roles to the high- and low-affinity Ca^{2+} binding sites, as, for example, high- and low-affinity forms of a Ca^{2+}-carrier protein. This would require in part, a demonstration of the interconvertability of the two sites. It is interesting to note that Worsfold and Peter (1970) have observed biphasic kinetics in Ca^{2+} transport, but the full significance of this observation and its relationship to the presence of two Ca^{2+}-specific membrane sites must await further study.

Our results on the effects of the lanthanide ions on Ca^{2+} binding are of particular interest. Because of their high charge

density, these ions should have a much greater electrostatic attraction for any negatively charged binding site than Ca^{2+} itself (Lettvin *et al.*, 1964). Indeed, a comparison of the stability of chelates of EDTA with various cations shows that the lanthanides form complexes about 10^8 times more stable than does Ca^{2+} (Dwyer and Mellor, 1964), reflecting the greater charge interaction of the $EDTA^{4-}$ anion with La^{3+} than with Ca^{2+}. Nevertheless, the lanthanides are relatively poor inhibitors of Ca^{2+} binding. Size factors alone probably do not account for this discrepancy. Values for the ionic radii of the lanthanides range from 0.858 Å for Yb^{3+} to 1.061 Å for La^{3+}, with Gd^{3+} (0.938 Å) very close to that of Ca^{2+} (0.94 Å) (Cotton and Wilkinson, 1966). Taken togehter, these data would suggest that charge interactions of the EDTA type are not of primary importance for Ca^{2+} binding to the membrane. Consistent with our results are the findings of Entman *et al.* (1969) which show that La^{3+} at 10^{-5} M does not affect Ca^{2+} accumulation, exchange, or the Ca^{2+}-stimulated ATPase in dog cardiac microsomes. In this respect it appears that the specificity for Ca^{2+} in the mitochondrial ion transport system differs significantly from that of the sarcoplasmic reticulum since a 50% inhibition of Ca^{2+} accumulation in rat liver mitochondria can be observed by lanthanides in the presence of more than a 3000-fold excess of Ca^{2+} (Mela and Chance, 1969; Mela, 1969).

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