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INTERACTION OF CATIONS AND LOCAL ANESTHETICS WITH ISOLATED SARCOLEMMA

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SUMMARY

Fragmented sarcolemma isolated from rabbit skeletal muscle bind Ca^{2+}, Mg^{2+} and Zn^{2+}. The capacity of sarcolemma for Ca^{2+} and Mg^{2+} is about 40 nmoles/mg of protein whereas for Zn^{2+} it is about 120 nmoles/mg of protein. From a mass law treatment we calculated the dissociation constants for the complex sarcolemma–cation as being 40 μM for Ca^{2+} and 43 μM for Mg^{2+}. For Zn^{2+} two different components for the binding were found and their estimated dissociation constants are 1.8 and 5 μM. Mg^{2+} competes with Ca^{2+} for the same binding sites and the binding of Ca^{2+} is inhibited very efficiently by Zn^{2+}.

The cations, Ca^{2+}, Mg^{2+} and Zn^{2+}, release H^+ from the sarcolemma such that the ratio of H^+ released to Ca^{2+} or Mg^{2+} bound is about 2.0 and that of H^+ released to Zn^{2+} bound varies between 1.0 and 1.5 depending on the concentration of Zn^{2+}.

The local anesthetics, quinine, tetracaine, procaine and chlorpromazine, inhibit the binding of Ca^{2+} by sarcolemma. Quinine, tetracaine and procaine act as competitive inhibitors, while chlorpromazine seems to be a non-competitive type of inhibitor. The order of inhibition by the drugs is chlorpromazine > quinine > tetracaine > procaine.

The alkaloid caffeine neither displaces the Ca^{2+} bound nor induces the release of H^+ by sarcolemma.

INTRODUCTION

Contraction of the skeletal muscle cell depends on the electrical activity of the sarcolemma[1, 2]. The electrical activity induces a series of events that leads to the liberation of Ca^{2+} normally bound by sarcoplasmic reticulum so that the Ca^{2+} may activate the contractile elements of the muscle cell[3,4]. The electrical activity of the excitable membrane depends on changes in its permeability to Na^+ and K^+ [5–7], and Ca^{2+} plays an important role in regulating the permeability and electrical activity of biological membranes[8,9]. Thus, the study of the interaction of Ca^{2+} with biological membranes, and in this particular case with sarcolemma isolated from skeletal muscle, is of extreme biological importance.

Furthermore, the study of membrane materials as cation exchangers is of ex-

treme importance in determining the physiological properties of the cell membrane since the isolated components of membranes, particularly the phospholopids and proteins, act as ion exchangers[10, 11]. The interaction of the phospholipids and lipoproteins of membrane materials with cations has been studied extensively[12–14], but only preliminary studies with isolated sarcolemma have been reported[15].

We investigated the Ca^{2+}, Mg^{2+} and Zn^{2+} binding to isolated sarcolemma, and the studies of the Ca^{2+} binding were extended to include an investigation of the effect of local anesthetics on the binding of this cation by the sarcolemma. Both Ca^{2+} and local anesthetics are membrane stabilizers although their actions differ in detail[16, 17]. Although local anesthetics may bind at the same sites as Ca^{2+} does, they can not substitute for Ca^{2+} with respect to the generation of the action potential[14]. We demonstrate in the present study that local anesthetics have the ability to displace Ca^{2+} bound from the isolated skeletal muscle sarcolemma. Their potency as Ca^{2+} displacers is related to their ability to release H^+ from the anionic groups of the membrane and to their potency as local anesthetics[17].

MATERIALS AND METHODS

Preparation of the biological material

Sarcolemma was isolated from the back and limb muscles of rabbit by the method of Rosenthal and co-workers[18] as modified by us. We started with about 150 g of muscle and the material collected from the 0.25 μM NaOH extracts was treated with 0.6 M KCl to remove the actomyosin. Furthermore, the fragments of sarcolemma were washed several times with cold deionized water until 3–4 l of water were used.

The final sediment, which consists of sarcolemma tubes, was homogenized in a glass–teflon homogenizer at 1000 rpm with 30–40 pestle strokes. The homogenate was washed three times with 100 ml of cold deionized water, centrifuged at 2000 $\times$ g for about two min in a table centrifuge and resuspended in water.

Typically it is possible to obtain 50–70 mg of sarcolemmal protein (biuret method[19]) from 150 g of muscle which represents a considerable improvement in yield over the original method of Rosenthal[18].

The material collected, when observed with a phase contrast microscope, consists of fragments of membranous material, pieces of sarcolemmal tubes, but no myofibrils are detected. Furthermore, the Mg-ATPase activities of the sarcolemmal fraction and that of the actomyosin gel obtained from the 0.6 M KCl extracts[44] during the process of isolation of the sarcolemma, show a different Ca^{2+} dependence (Fig. 1). Thus, the ATPase activity of actomyosin reaches a maximal value at a pCa value of 6–5, while the ATPase of sarcolemma exhibits a maximum at pCa of 3.5. Furthermore, the Ca^{2+} concentration that induces a maximum ATPase for actomyosin observed in our experiments is lower than that commonly observed, and this may be due to the incubation at 37 °C we made during the isolation of sarcolemma that may partially denaturate the enzyme.

We also studied the Ca^{2+} binding concomitantly in some sarcolemmal preparations and in the actomyosin removed during the preparation of the sarcolemma. The Ca^{2+} bound by actomyosin is very low when compared with the amount of ion bound by the sarcolemma. For a free Ca^{2+} concentration of 100 μM, actomyosin binds 3–4 nmoles/mg protein, while sarcolemma binds about 25 nmoles/mg protein (Fig. 2).

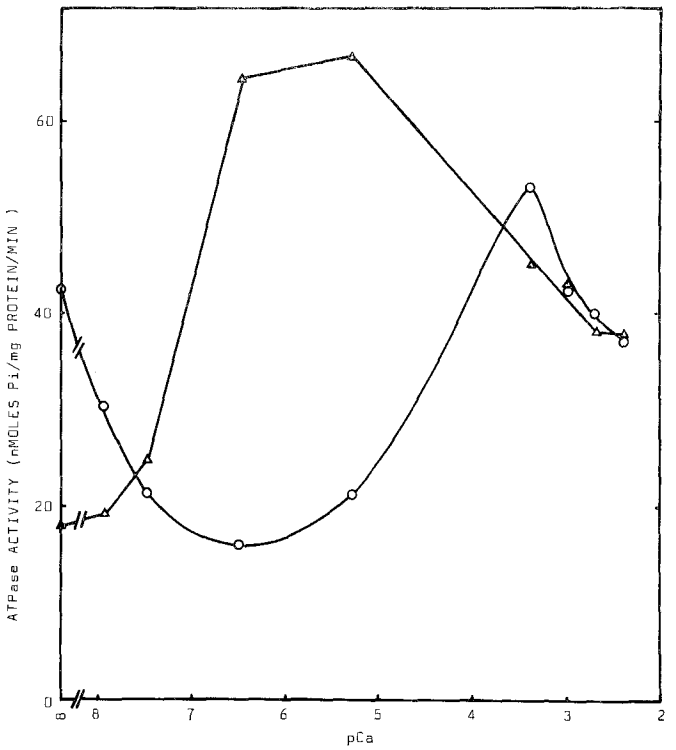

Fig. 1. Adenosinetriphosphatase activities of sarcolemma (○—○) and actomyosin (△—△). The dependence on Ca^{2+} of the Mg-ATPases of the sarcolemma, and of the actomyosin which was extracted during the preparation of sarcolemma, is depicted. The experiments were performed at 20 °C and at pH 7.3 with constant stirring. The medium contained 2.5 mg of sarcolemmal protein or actomyosin and 5 mM Tris, 30 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, 1 mM EGTA and $CaCl_2$ in the desired concentrations (total volume 5 ml). The concentration of free Ca^{2+} was calculated taking 10^{-11} as dissociation constant[43] for CaEGTA, corrected for pH of 7.3. The reaction was stopped with 1 ml 20% trichloroacetic acid before 10% of the total ATP was hydrolized. Suspensions were filtered through Whatman filters No 41 and the inorganic phosphate in the clear filtrates was determined by the method described by Taussky and Shorr[24].

Cation binding by sarcolemma

Suspensions of sarcolemma containing 1.5 mg protein were incubated in preweighed polycarbonate tubes for 15 min at 20 °C with stirring in a magnetic device, in a medium containing 4 mM imidazole at pH 6.9 and substances in the desired concentrations (total volume 5 ml). The suspensions were centrifuged at 3000 × *g* for 12 min in a table centrifuge and the supernatant solutions were transferred to another series of tubes and kept for cation analysis. The tubes containing the pellets were further decanted as completely as possible, droplets of supernatant were absorbed with filter paper and the tubes were weighed. The weight of sediments was calculated and was taken to represent approximately the weight of the supernatant portion that could not be removed by discarding the supernatants.

The residues in the tubes were extracted in 10 % trichloroacetic acid, and the resulting suspensions were centrifuged as above. The amounts of cations in the trichloroacetic acid extracts were measured by atomic absorption spectrophotometry using an AC 2-20 Bausch and Lomb absorption spectrophotometer. The concentra-

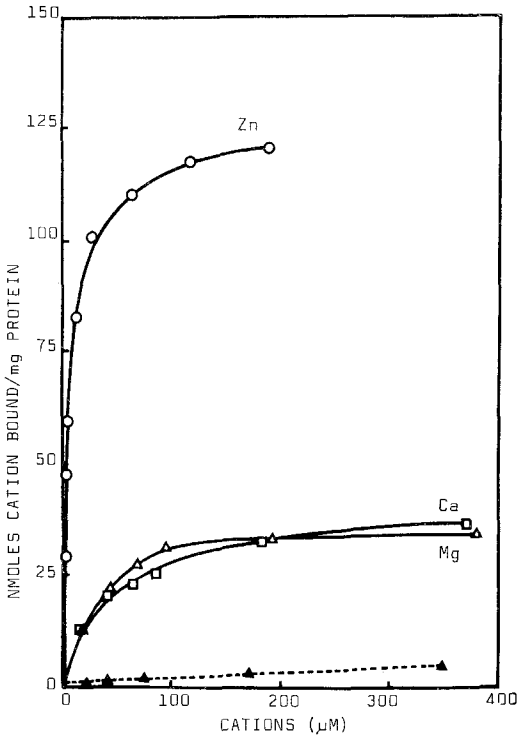

Fig. 2. Binding of Ca^{2+}, Mg^{2+} and Zn^{2+} by the sarcolemma isolated from rabbit skeletal muscle Sarcolemma fragments (1.5 mg protein) were incubated for 15 min at 20 °C in a medium containing 4 mM imidazole at pH 6.9 and cations in the desired concentration (total volume 5 ml). The cations bound and free were measured by atomic absorption spectrophotometry. The dashed line (▲---▲) represents the binding of Ca^{2+} by actomyosin extracted during the preparation of the sarcolemma. On the abscissa are represented the concentrations of free cations in equilibrium with protein.

tions of the free cations in equilibrium with the sarcolemma were measured in the supernatant solutions of the first centrifugation. For determinations of calcium concentrations, 1 % $LaCl_3$ was added to the standards and to the samples to prevent interferences; for determinations of Mg^{2+} concentrations, 1 % of $SrCl_2$ was added[20].

The amount of cation remaining in the supernatant trapped in the pellets was subtracted from the total amount of cation present in the trichloroacetic acid extract of membranes. This correction usually represented a small fraction of the cations retained in the pellets (about 1–9 %) since the maximal concentration of free cation used was only 400 μM.

Preparation of lipid free sarcolemma

The lipids were extracted from sarcolemma essentially by the method of Fleischer[21]. After the extraction, the suspension was centrifuged at 2000 × g for 2 min, and the sedimented material was washed once with 100 ml cold water and resuspended in water. Protein analyses were made before and after extraction of the lipid. The amount of phospholipid present before and after the extraction, was determined by a modification of the method of Schneider[22,23]. The inorganic phosphate

was analyzed in the digested samples by the method of Taussky and Shorr[24]. The amount of phospholipid in the original membranes was calculated by multiplying the weight of inorganic phosphate by a factor of 25 (ref. 25). About 95 % of the phospholipid of the sarcolemma was removed by the acetone–NH_3 extraction.

H^+ release by sarcolemma

Aqueous suspensions of sarcolemma containing 4–5 mg of protein were adjusted to pH 7.0 by adding 10 mM KOH. Afterwards we studied the effect of various cations and drugs on the H^+ release by sarcolemma. The substances were added with a syringe microburet (Model No SB2 Micro-metric Instrument Co., Cleveland-Ohio) and we then recorded the amounts of 10 mM KOH delivered from the autoburette of an automatic Radiometer pH stat in order to maintain the pH at 7.0.

Reagents

All reagents used were of reagent grade. Imidazole, quinine–HCl, procaine-HCl were obtained from Sigma Chemical Co., St. Louis; tetracaine–HCl from KK laboratories, Plainview, New York, and chlorpromazine–HCl from Smith Kline and French Laboratories, Philadelphia.

RESULTS

Binding of cations by sarcolemma

The binding of Ca^{2+}, Mg^{2+} and Zn^{2+} was studied. The amounts of Ca^{2+}, Mg^{2+} and Zn^{2+} bound per mg of protein were plotted against the concentration of free cation in equilibrium with sarcolemma fragments. From the plots obtained we can see that saturation of the binding sites of sarcolemma occurs when concentration of free Ca^{2+}, Mg^{2+} or Zn^{2+} approaches 100–200 μM (Fig. 1). The sarcolemma has a maximal binding for Ca^{2+} or Mg^{2+} of about 40 nmoles/mg of protein and a Zn^{2+} maximal binding capacity of about 120 nmoles/mg of protein (Fig. 2).

The results were treated according to the mass law, assuming that the binding sites in the membrane complex the cation and that the cations complexed are in equilibrium with free cations in the medium. It was also assumed that the binding sites are independent and equivalent. The dissociation of the complex can be represented by the equation:

$$SMe \rightleftharpoons S^- + Me^+ \qquad (1)$$

It should be possible to treat the data obtained according to the mass law as it has been done in other membrane systems[26,27] using the following equation:

$$\frac{1}{b} = \frac{K_D}{B_{max}} \cdot \frac{1}{(Me^+)} + \frac{1}{B_{max}} \qquad (2)$$

where b is the amount of cation bound, B_{max} is the maximum binding and K_D the dissociation constant for the sarcolemma–cation complex. Plotting the results obtained for binding of the cations on the basis of Eqn 2 we calculated the dissociation constant and maximum binding of the sarcolemma for each cation. Straight lines were obtained

for the binding of Ca^{2+} and Mg^{2+} suggesting that only one type of sites or different but equivalent sites are involved in the binding (Fig. 3).

The plot of the data obtained with Zn^{2+} can be resolved into two straight lines, which suggests that two classes of binding sites are involved (Fig. 4B). An equation for the case of two different types of binding sites can be obtained from Eqn 2[13,28]:

$$\frac{b_t}{(Me^+)} = \frac{B_{max_1}}{K_{D_1}} + \frac{B_{max_2}}{K_{D_2}} - \frac{b_1}{K_{D_1}} - \frac{b_2}{K_{D_2}} \qquad (3)$$

where b_t is the total cation bound, B_{max_1} and B_{max_2} are the maximum binding capaci-

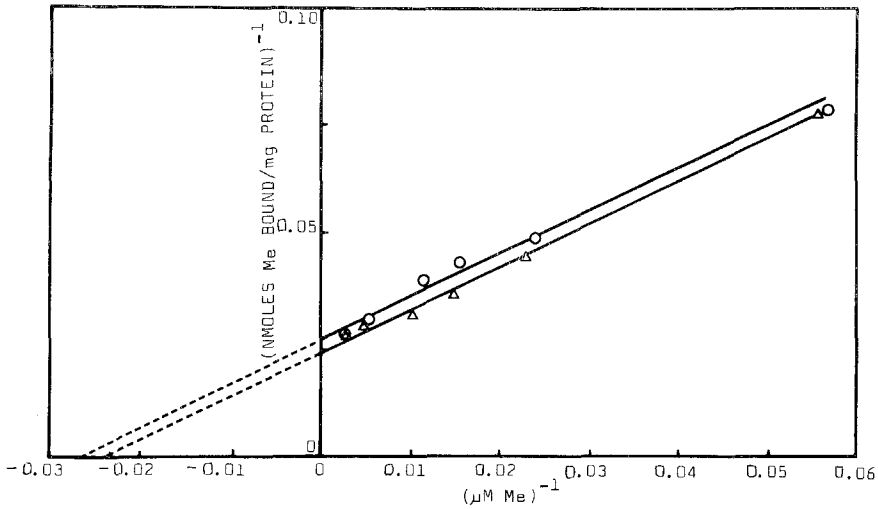

Fig. 3. Reciprocal plot for the binding of the metals (Me) Ca^{2+} (○—○) and Mg^{2+} (△—△). The intercept at the ordinate represents the reciprocal of the maximum binding. The intercept at the abscissa represents the negative of the reciprocal of the apparent dissociation constant for the sarcolemma–Me complex.

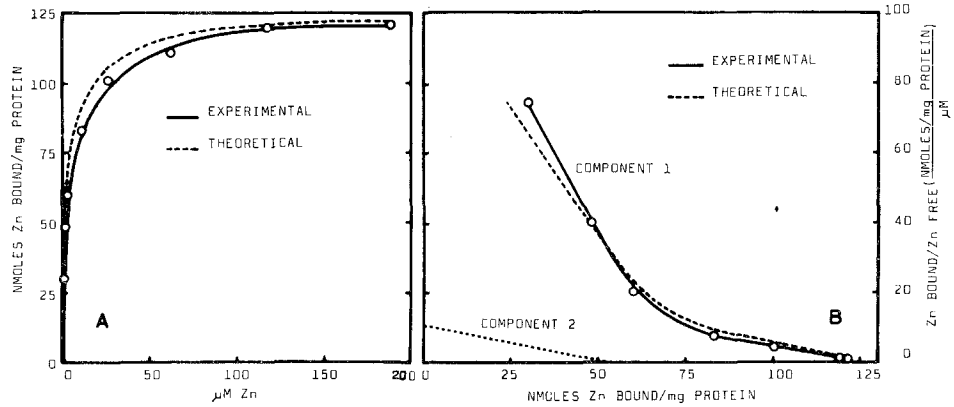

Fig. 4. A. Binding of Zn^{2+} by the isolated sarcolemma. The dashed line represents the theoretical curve as calculated following the model proposed for the binding of Zn^{2+} by two distinct classes of binding sites. B. Scatchard type of plot for the binding of Zn^{2+} by the sarcolemma. The curve was resolved into two components. The dashed line is a theoretical curve calculated following the model proposed for the binding of Zn^{2+}.

ties of the two types of binding sites, K_{D_1} and K_{D_2} are the respective dissociation constants, b_1 and b_2 are the amounts of cation bound to each of the binding sites.

By making certain aproximations[13, 28], it was possible to calculate B_{max_1}, B_{max_2}, K_{D_1} and K_{D_2}. With these estimated values we could obtain theoretical curves that approach the experimental ones (Fig. 4A, B). The values for the dissociation constants and maximum binding capacities are summarized in Table I.

Competitive binding studies

In these studies reciprocal values of Ca^{2+} bound by sarcolemma were plotted against the concentrations of the substances which inhibited Ca^{2+} binding. The values obtained with two concentrations of Ca^{2+} were plotted. The intersection of the two straight lines obtained has the following significance: If the intersection occurs above the abscissa the inhibition is competitive, whereas if the intersection is at the abscissa, the inhibition is of the non-competitive type[29]. From the results obtained we can see that Mg^{2+} inhibits competitively the binding of Ca^{2+} by sarcolemma (Fig. 5A), and Zn^{2+} has an inhibitory effect greater than Mg^{2+} (Fig. 5B). The estimated apparent

TABLE I

CONSTANTS FOR CATION BINDING BY RABBIT SKELETAL MUSCLE SARCOLEMMA AS DETERMINED FROM A MASS LAW TREATMENT

Cation	*Maximum binding capacity (nmoles/mg protein)*	*Dissociation constant (μM)*
Ca^{2+}	35.6 ± 3.6 (5)	40.20 ± 3.92 (5)
Mg^{2+}	36.8 ± 3.5 (5)	43.84 ± 4.20 (5)
Zn^{2+}	$B_{max_1} = 70.2 \pm 5.2$ (4)	$K_{D_1} = 1.81 \pm 0.18$ (4)
	$B_{max_2} = 52.1 \pm 4.7$ (4)	$K_{D_2} = 4.95 \pm 0.43$ (4)

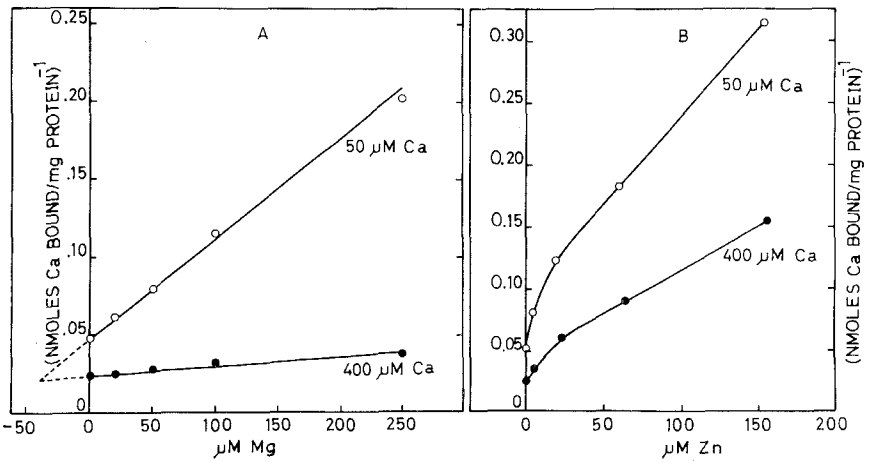

Fig. 5. A. Effect of Mg^{2+} on the binding of Ca^{2+} by sarcolemma. A competitive type of inhibition is observed for Mg^{2+} since the intersection of the straight lines obtained at two concentrations of Ca^{2+} occurs above the abscissa. The abscissa value for the point of intersection gives the apparent constant for the inhibitor. B. Effect of Zn^{2+} on the binding of Ca^{2+} by sarcolemma. The type of interaction is not clear at the moment since straight lines can not be obtained. However, it can be seen that Zn^{2+} inhibits very efficiently the binding of the Ca^{2+}.

inhibition constant (K_i) for Mg^{2+} is about 40 μM Mg^{2+}, which is very close to the value of K_D determined for the binding of Mg^{2+} (43.8 μM). The inhibitory effect of Zn^{2+} is not yet clear since Zn^{2+} has at least two types of binding sites. In current studies we are attempting to obtain more information to characterize the inhibition.

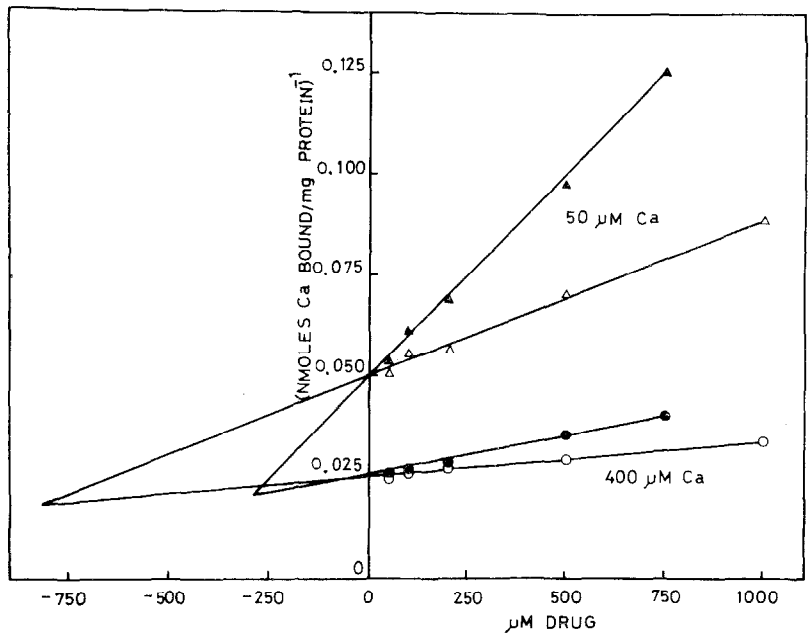

Fig. 6. Interaction of quinine (● and ▲) and tetracaine (○ and △) on the binding of Ca^{2+} by the sarcolemma. These drugs inhibit the binding of Ca^{2+} competitively. The abscissa value at the point of intersection of the two straight lines gives the apparent inhibition constants of the drugs. Quinine has a more potent effect in displacing Ca^{2+} ($K_i = 275$ μM) than does tetracaine ($K_i = 800$ μM).

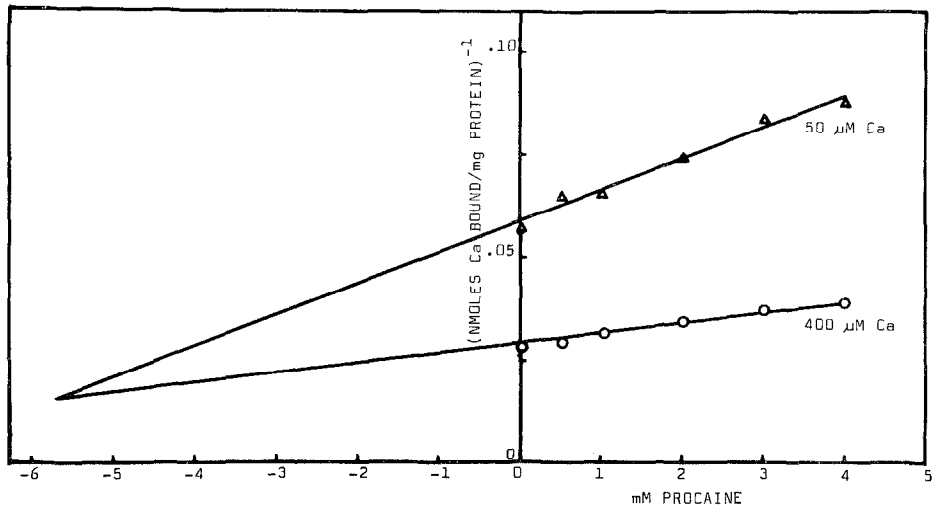

Fig. 7. Effect of procaine on the binding of Ca^{2+} by sarcolemma. This drug has the smallest effect in displacing Ca^{2+} as compared with the other local anesthetics we have studied. The estimated apparent dissociation constant for the sarcolemma–procaine complex is about 5.8 mM procaine.

The effects of some local anesthetics on Ca^{2+} binding by sarcolemma were also studied. Quinine, tetracaine and procaine inhibit competitively the Ca^{2+} binding by sarcolemma (Figs 6 and 7). Quinine has the greater inhibitory effect. Procaine has a small inhibitory effect and only relatively large concentrations of the anesthetic induce significant inhibition (Fig. 7). The apparent inhibition constants (K_i) are 275 μM for quinine (Fig. 6), 800 μM for tetracaine (Fig. 6) and 5.8 mM for procaine (Fig. 7).

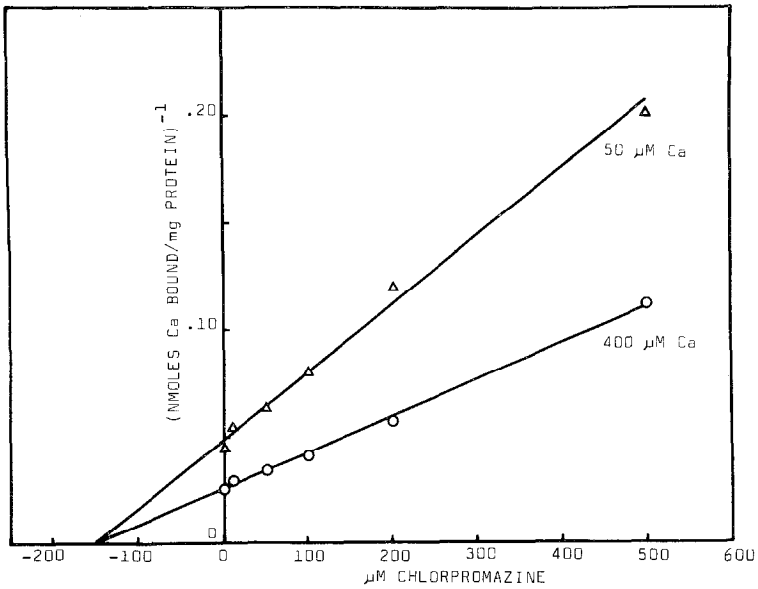

Fig. 8. Effect of chlorpromazine on the binding of Ca^{2+} by sarcolemma. This anesthetic inhibits the binding of the Ca^{2+} non-competitively. The estimated apparent inhibition constant was 150 μM chlorpromazine.

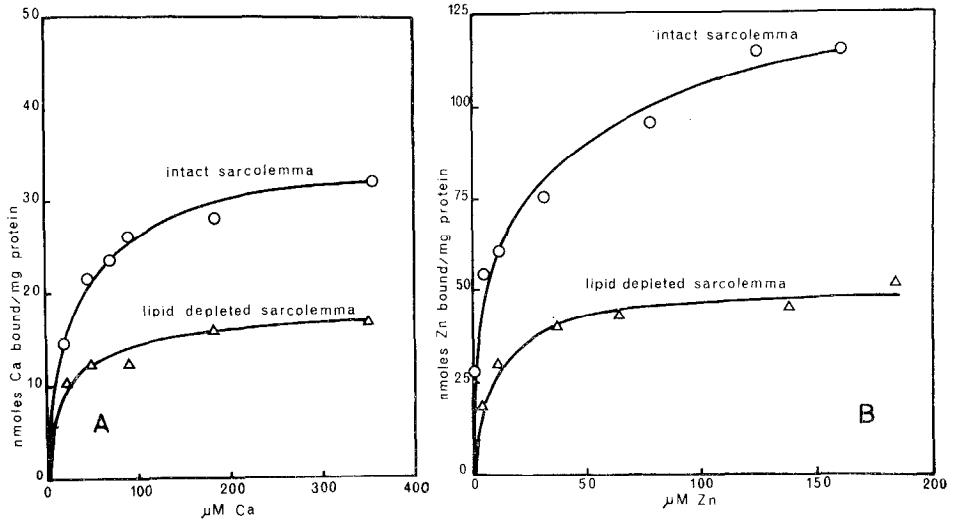

Fig. 9. Binding of Ca^{2+} (Fig. 9A) and of Zn^{2+} (Fig. 9B) by lipid-depleted sarcolemma as compared to the binding by intact sarcolemma at various concentrations of added Ca^{2+} or Zn^{2+}.

Similar studies with chlorpromazine (Fig. 8) suggest that this drug inhibits the binding of Ca^{2+} by sarcolemma like a non-competitive type inhibitor. The inhibitory effect of chlorpromazine is much greater than that of the drugs described above and its apparent inhibition constant is about 150 μM.

Binding of cations by lipid-free sarcolemma

Binding of both Ca^{2+} and Zn^{2+} was studied concurrently in intact and lipid-depleted sarcolemma. The amount of cations bound to acetone-treated sarcolemma was lower than the amount bound to intact membranes (Fig. 9A and B). The cation binding capacity of lipid-free sarcolemma is about 50 % of the binding capacity of the intact sarcolemma. These results suggest that the membrane lipids play an important role in the binding of the cations.

H^+ release by sarcolemma

The results of the effects of Ca^{2+}, Mg^{2+} and Zn^{2+} on H^+ release by sarcolemma are represented in Fig. 10. The amount of H^+ released by Ca^{2+} or Mg^{2+} is lower than that released by Zn^{2+}. At Zn^{2+} concentrations above 400–500 nmoles added per mg of protein the amount of H^+ released per unit of concentration of Zn^{2+} added is higher than at the lower concentrations of Zn^{2+}. At those concentrations of Zn^{2+}, flocculation of sarcolemmal protein is observed which may represent denaturation of the protein and, therefore, exposure of more binding sites which might release H^+. The unfolding process of the protein due to denaturation may cause exposure of ion pairs initially inside the folded form of the protein. The ion pairs now present in the hydrophylic medium may ionize.

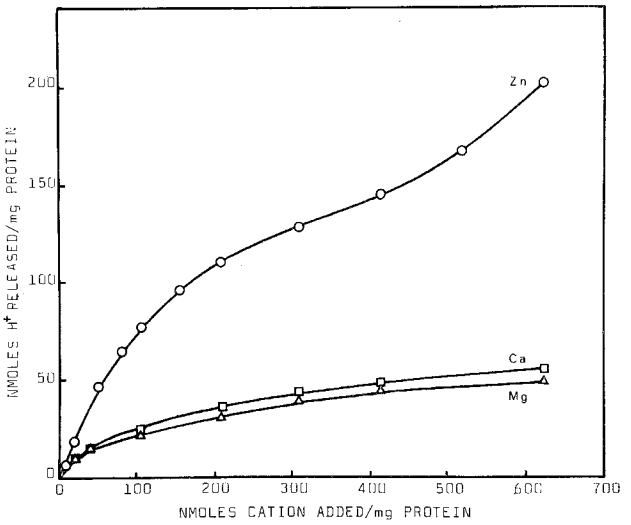

Fig. 10. Effect of Ca^{2+}, Mg^{2+} and Zn^{2+} on H^+ release by the sarcolemma. The H^+ release was studied at pH 7.0 by means of an automatic Radiometer pH stat as described in the methods. Ca^{2+} or Mg^{2+} release about equal amounts of H^+. Zn^{2+} induces an H^+ release much greater than do the other cations. At Zn^{2+} concentrations above 400–500 nmoles added per mg of protein, the H^+ released per Zn^{2+} added increases. Flocculation of the sarcolemmal material occurs at this point suggesting that denaturation occurs and, therefore, more binding sites which release H^+ might be exposed.

The dependence of the release of H^+ on some local anesthetics (chlorpromazine, quinine, tetracaine and procaine) was also studied. The effects of drugs were compared with the effects of Ca^{2+} on H^+ release (Fig. 11). Chlorpromazine induces an amount of H^+ release about 1.5 times greater than does Ca^{2+} for concentrations of 850 nmoles of

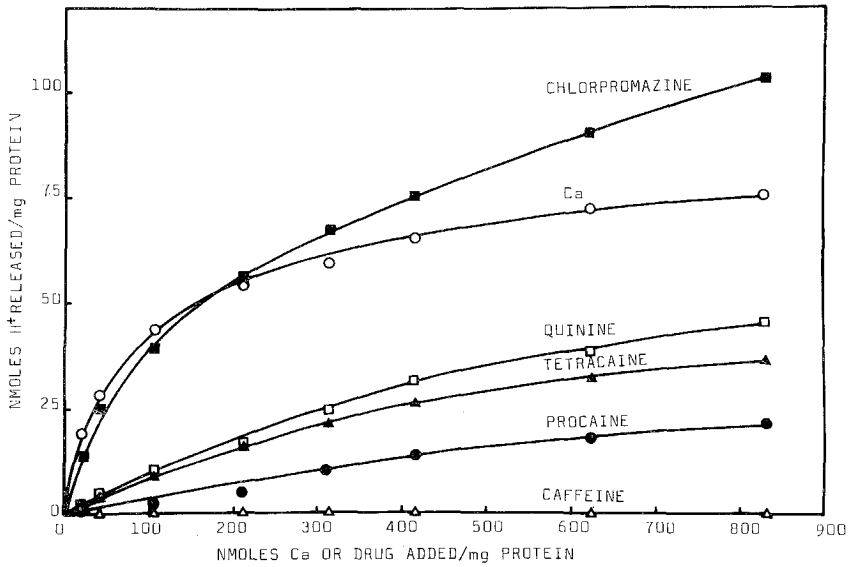

Fig. 11. Hydrogen-ion release induced by the local anesthetics, chlorpromazine, quinine, tetracaine and procaine. The effect of these drugs on the release of H^+ is compared with the effect of Ca^{2+}. The order of H^+-releasing efficiency of the drugs is chlorpromazine > quinine > tetracaine > procaine. Caffeine has no effect on the H^+ release.

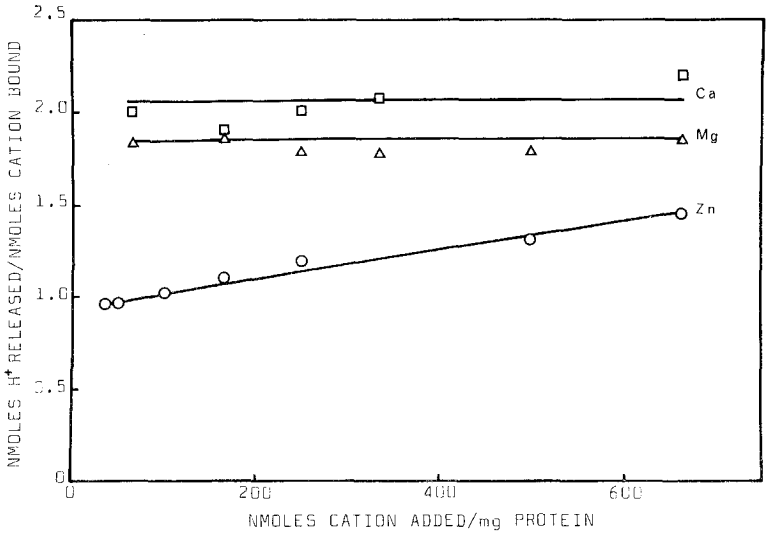

Fig. 12. Cation binding and H^+ release by sarcolemma. The ratio between H^+ released and cation bound is plotted as a function of the amount of cation added to the medium. The value for the ratio is about 2.0 for Ca^{2+}, about 1.8 for Mg^{2+} and 1.0 to 1.5 for Zn^{2+}.

each substance added per mg of protein. For low concentrations of added substance (0–170 nmoles/mg protein) chlorpromazine has about the same effect on H^+ release as does Ca^{2+} (Fig. 11).

The studies with quinine and tetracaine reveal that for high concentrations added (850 nmoles/mg protein), these substances induce only about 50 % of H^+ release by sarcolemma when compared with the effect of Ca^{2+}. For lower concentrations, the ratio of H^+ released by Ca^{2+} to the H^+ released by the drugs is increased, and the maximal value is about 4.5 when 100 nmoles of Ca^{2+} or drug is added per mg of protein (Fig. 11). Furthermore, the results obtained suggest that quinine is slightly more efficient in releasing H^+ by sarcolemma than is tetracaine. On the other hand, the action of procaine in releasing H^+ by sarcolemma is only about 0.5 and 0.25 as effective as respectively the action of quinine or Ca^{2+}.

Studies with caffeine (Fig. 11) indicate that no release of H^+ occurs when this substance is added to sarcolemma suspensions.

Cation binding and H^+ release

We studied the relationship between the binding of Ca^{2+}, Mg^{2+} and Zn^{2+}, and the dependence of H^+ release on the binding of these ions. A plot of the ratio between H^+ released and cation bound against the amount of cation added to sarcolemma preparations shows that a constant ratio of about 2.0 H^+ are released per Ca^{2+} bound and 1.8 H^+ are released per Mg^{2+}. For the case of Zn^{2+}, the value varies between about 1.0 at low concentration of Zn^{2+} in the medium and about 1.5 at the higher concentrations (Fig. 12).

DISCUSSION

Binding of cations and the release of H^+

In the present study we demonstrated that sarcolemma has the ability to bind ions such as Ca^{2+}, Mg^{2+} and Zn^{2+}. The binding of cations seems to be characteristic of most biological membranes since it has been demonstrated in sarcoplasmic reticulum[12,30], mitochondrial membranes[27,31], renal cortex membranes[33] and many other materials. The binding of Ca^{2+} by the sarcolemma has been demonstrated in a preliminary study by Koketsu *et al.*[15]. The binding of cations probably involves interactions with membrane anionic groups like phosphoric sites of phospholipids and carboxyl groups of protein. In agreement with other authors[27,31], our results indicate that membrane lipids, probably phospholipids, are largely involved in cation binding, since when phospholipids are removed the amount of cations bound decreases by a large amount (Fig. 8A, B).

It seems that Ca^{2+} and Mg^{2+} bind to sites initially occupied by H^+ since the ratio between H^+ released and ions bound is about 2.0. It is reasonable to think that each Ca^{2+} or Mg^{2+} binds to two acid groups of the membrane. The binding of Zn^{2+} is more difficult to characterize. In fact, the amount of Zn^{2+} bound by the sarcolemma is much greater when compared with that of Ca^{2+} or Mg^{2+}, and the binding seems more complicated, and will require further study to permit an interpretation. However, it appears reasonable to think that most of the Zn^{2+} binds to acid groups since the amount of H^+ released by Zn^{2+} is greater than that released by Ca^{2+} or Mg^{2+} at the same concentrations of the cations in agreement with the higher capacity of mem-

brane binding sites for Zn^{2+} than for Mg^{2+} or Ca^{2+}. The high capacity for Zn^{2+} has been demonstrated in other membrane materials[12,31,32].

The results of our studies suggest that Mg^{2+} and Ca^{2+} bind at the same sites of the sarcolemma. We demonstrated that sarcolemma has about the same affinity for Ca^{2+} or for Mg^{2+} and that Mg^{2+} inhibits the binding of Ca^{2+} competitively. A similar type of competition has been reported for the sarcoplasmic reticulum[12], heart mitochondrial membranes[31] and to submitochondrial particles[27].

The role of Zn^{2+} as a potentiatior of muscle contraction[34,36] led us to investigate the effect of this ion on the binding of Ca^{2+} by the sarcolemma. Zn^{2+} inhibits very effectively the binding of Ca^{2+} which may be related to the action of Zn^{2+} on muscular contraction. This action has in fact been shown to result from the interaction of Zn^{2+} with the surface of the muscle cell which prolongs the repolarization phase of the action potential[34,36]. Since Ca^{2+} plays an important role in controlling the activity of the excitable membranes[8,37,38], the binding of Zn^{2+} to the sarcolemma in exchange for Ca^{2+} may alter the permeability properties of the plasma membranes and induce alterations in its electrical properties. This may account for the potentiation effect of Zn^{2+} (refs 34 and 35).

Effect of local anesthetics on Ca^{2+} binding and on H^+ release by sarcolemma

We demonstrated that local anesthetics like quinine, tetracaine and chlorpromazine can displace the Ca^{2+} bound by the sarcolemma. Procaine also shows this effect, but for this drug it is much smaller than that of the others. These drugs inhibit the Ca^{2+} binding competitively except for chlorpromazine which acts as a non-competitive inhibitor. It seems that these drugs bind to the same sites that bind Ca^{2+}. These sites probably are the anionic groups of phospholipids and proteins that were initially associated with H^+. There is good agreement between the potency of the local anesthetic to displace Ca^{2+} and their ability to release H^+ in the absence of Ca^{2+}. The more potent is the drug with respect to its inhibition of Ca^{2+} binding, the more potent is its effect in releasing H^+

The displacement of Ca^{2+} by local anesthetics has been demonstrated in other membrane materials like muscle microsomes[30,39], submitochondrial particles[27], red blood cell membranes[40] and lobster giant axon[41]. Chlorpromazine inhibits very efficiently the binding of the Ca^{2+}, but acts as a non-competitive inhibitor. This result disagrees with that found by Kwant and Seeman[40] who reported for red cells a competitive inhibitory effect of the drug with respect to the binding of Ca^{2+}. However, our material is different in origin and different mechanisms may be involved in the two cases.

The local anesthetics bind to phospholipids and membrane lipoproteins at the same sites that bind Ca^{2+}[14]. This is suggested by the stabilizing effect of both calcium and local anesthetics on the squid giant axon[16] and on lobster giant axon[41]. The strong interaction of Ca^{2+} and local anesthetics with isolated sarcolemma observed in our study suggests that the action of these substances on the muscle cell membrane may follow a similar mechanism to that proposed for the membranes of squid[16] and lobster[41] giant axons.

Caffeine in concentration of 0–1 mM does not displace the Ca^{2+} bound to sarcolemma and does not release H^+. Thus caffeine does not compete with Ca^{2+} for the anionic groups of rabbit muscle sarcolemma. Carvalho[12] arrived at the same conclu-

sion for the sarcoplasmic reticulum. These observations do not support earlier postulates that caffeine displaces the Ca^{2+} bound to the sarcolemma or sarcoplasmic reticulum[42]. It is more reasonable to think that the potentiating effect of caffeine is related to the release of a fraction of Ca^{2+} which is not in the bound form as proposed earlier[23].

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REFERENCES

1 A. Sandow, *Pharmacol. Rev.*, 17 (1965) 265.
2 A. Sandow, S. R. Taylor and H. Preiser, *Fed. Proc.*, 24 (1965) 1116.
3 S. Ebashi and M. Endo, *Prog. Biophys. Mol. Biol.*, 18 (1968) 125.
4 A. Weber, R. Herz and I. Reiss, *Proc. Roy. Soc. London Ser. B*, 160 (1964) 489.
5 A. L. Hodgkin and A. F. Huxley, *Cold Spring Harbor Symp. Quant. Biol.*, 17 (1952) 43.
6 A. L. Hodgkin and A. F. Huxley, *J. Physiol.*, 117 (1952) 500.
7 W. L. Nastuk and A. L. Hodgkin, *J. Cell. Comp. Physiol.*, 35 (1950) 39.
8 K. Koketsu and S. Miyamoto, *Proc. 23rd. Int. Congr. Physiol. Sci., Int. Congr. Series*, 87 (1965) 521.
9 J. F. Manery, *Fed. Proc.*, 25 (1966) 1804.
10 M. B. Abramson, R. Katzman and H. Gregor, *J. Biol. Chem.*, 239 (1964) 70.
11 H. L. Rosano, P. Duby and J. H. Schulman, *J. Phys. Chem.*, 65 (1961) 1704.
12 A. P. Carvalho, *J. Cell. Comp. Physiol.*, 67 (1966) 73.
13 A. P. Carvalho, H. Sanui and N. Pace, *J. Cell. Comp. Physiol.*, 66 (1965) 57.
14 M. B. Feinstein, *J. Gen. Physiol.*, 48 (1964) 357.
15 K. Koketsu, R. Kitamura and R. Tanaka, *Am. J. Physiol.*, 207 (1964) 509.
16 A. M. Shanes, W. H. Freygang, H. Grundfest and E. Amatniek, *J. Gen. Physiol.*, 42 (1959) 793.
17 A. W. Cuthbert, *Pharmacol. Rev.*, 19 (1967) 59.
18 S. L. Rosenthal, P. M. Edelman and I. L. Schwartz, *Biochim. Biophys. Acta*, 109 (1965) 512.
19 A. G. Cornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.*, 177 (1949) 751.
20 J. B. Willis, in *Methods of Biochemical Analysis*, Vol. 3, John Wiley and Sons, Inc., New York, 1963.
21 S. Fleischer, B. Fleischer and W. Stoeckenius, *J. Cell Biol.*, 32 (1967) 193.
22 W. C. Schneider, *J. Biol. Chem.*, 161 (1945) 293.
23 A. P. Carvalho and B. Leo, *J. Gen. Physiol.*, 50 (1967) 1327.
24 H. H. Taussky and E. Shorr, *J. Biol. Chem.*, 202 (1953) 675.
25 H. Wittcoff, *The Phospholipids*, Reinholds Publishing Corp., New York, 1951.
26 H. Sanui and N. Pace, *J. Gen. Physiol.*, 42 (1959) 1325.
27 A. Scarpa and A. Azzi, *Biochim. Biophys. Acta*, 150 (1968) 473.
28 H. Sanui, A. P. Carvalho and N. Pace, *J. Cell. Comp. Physiol.*, 59 (1962) 241.
29 H. R. Mahler and E. H. Cordes, *Biological Chemistry*, Harper and Row, Inc., New York, 1966.
30 A. P. Carvalho, *J. Gen. Physiol.*, 52 (1968) 622.
31 W. E. Jacobus and G. P. Brierley, *J. Biol. Chem.*, 244 (1969) 4995.
32 A. P. Carvalho, *Eur. J. Biochem.*, in the press.
33 R. F. Palmer and V. A. Posey, *J. Gen. Physiol.*, 55 (1970) 89.
34 A. Sandow, *Arch. Phys. Med. Rehabil.*, 45 (1964) 62.
35 A. Sandow, S. R. Taylor, S. Isaacson and J. J. Seguin, *Science*, 143 (1964) 577.
36 H. Mashima and H. Washio, *Jap. J. Physiol.*, 14 (1964) 538.
37 B. Frankenhaeuser and A. L. Hodgkin, *J. Physiol.*, 137 (1957) 218.
38 K. Koketsu and S. Miyamoto, *Nature*, 189 (1961) 402.
39 A. Bondani and R. Karler, *J. Cell. Comp. Physiol.*, 75 (1970) 199.
40 W. O. Kwant and P. Seeman, *Biochim. Biophys. Acta*, 193 (1969) 338.
41 M. P. Blaustein and D. E. Goldman, *J. Gen. Physiol.*, 49 (1966) 1043.
42 G. B. Frank, *J. Physiol.*, 163 (1962) 254.
43 S. Chaberek and A. E. Martell, *Organic Sequestring Agents*, Wiley and Sons, Inc., New York, 1959.
44 S. Ebashi, *J. Biochem.*, 50 (1961) 236.