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CALCIUM ION BINDING TO LOBSTER NERVE MEMBRANES

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Summary

The binding of $^{45}Ca^{2+}$ to membrane material isolated from lobster walking leg nerves was studied using a rapid filtration technique. In solutions of high ionic strength (450 mM), the amount of $^{45}Ca^{2+}$ bound to this membrane material was found to be highly dependent on the monovalent cation used in the incubating solution. The amount of $^{45}Ca^{2+}$ bound was larger when the membranes were incubated in a KCl solution compared to when they were incubated in a NaCl solution. This difference was attributed to the ability of these closed membrane vesicles to accumulate Ca^{2+} into the vesicle when incubating in a KCl solution but not in a NaCl solution. This accumulation of Ca^{2+} was found to be independent of metabolic energy and depended primarily on the absence of Na^+ from the incubation medium. At low ionic strength, the membranes formed open fragments and the amount of Ca^{2+} bound was no longer sensitive to the monovalent cation species in the incubation solution. The $^{45}Ca^{2+}$ bound under these low ionic strength conditions was considered to be bound to anionic sites on the membranes.

Introduction

Although it is widely accepted that Ca^{2+} plays an important role in nerve conduction, its mechanism of action is still not entirely understood. It has been proposed [1] that nerve membranes have monovalent/divalent cation exchanger properties associated with their external surface. According to this hypothesis, these anionic membrane sites are occupied predominantly by Ca^{2+} in the resting state of the nerve and mainly by monovalent cations in the excited state (depolarized state) of the nerve. Although much electrophysiological data has been published in support of this hypothesis [1,2], very few direct biochemical studies have been done aimed at examining this property of nerve

membranes. The purpose of this work was to characterize the binding of Ca^{2+} to these anionic membrane sites.

Previous investigations dealing with the binding of Ca^{2+} to membrane material are quite limited. Kamino et al. [3] have shown that Ca^{2+} binds to rat brain synaptosomes with a lower dissociation constant when the membranes were incubated in an isotonic NaCl solution compared to when they were incubated in a KCl solution. However, since these brain synaptosomes contain membranes whose primary function is in synaptic (chemical) transmission, rather than (electrical) nerve conduction, it may be inappropriate to draw conclusions on the nature of nerve conduction from their data. Lieberman et al. [4] found a metabolic energy dependent uptake of $^{45}Ca^{2+}$ in a preparation of crab nerve membrane. The relation between this finding and the cation-exchangers property of nerve membrane was, however, not studied. Abood has performed a number of interesting studies on the binding of Ca^{2+} to membrane lipids [5] and to membrane proteins from mammalian brain synaptosomes [6]. More recently, Baker and McNaughton [7] studied the effect of extracellular Ca^{2+}-binding on the Ca^{2+} efflux from squid axons. Although this last study did suggest the presence of a Ca^{2+}-binding matrix external to the plasma membrane, its relation to excitability is unclear.

In addition to binding to these external sites, Ca^{2+} has been shown to accumulate in nerve in response to a number of different processes, including depolarization [8] and alteration of both the internal and external Na^+ concentration [9,10]. Thus, data on the binding of Ca^{2+} to external sites is complicated by these various components of Ca^{2+} uptake.

In the present study, both the binding of Ca^{2+} to membrane sites and the accumulation of Ca^{2+} into vesicles formed by membranes from lobster nerve was studied. The accumulation of Ca^{2+} was found to be inhibited by Na^+ in the incubating solution by a mechanism similar to one previously described for intact nerve [9,10].

Methods

Membrane preparation

Nerve membranes were isolated by a method similar to that described by Denburg [11]. Briefly, this procedure consisted in removing the nerve bundles from the eight walking legs of lobsters (*Homarus americanus*), cutting them into small pieces and mixing the pieces with a solution containing 0.32 M sucrose and 10 mM Tris-HCl (pH 7.8). The nerves were then homogenized in a teflon-glass Potter-Elvehjem homogenizer using 10—15 up-and-down strokes of an electrically driven pestle. This crude homogenate was spun at $1000 \times g$ for 10 min in a tabletop centrifuge. The resulting supernatant was collected by decantation. The pellet was resuspended in the sucrose solution and again spun at $1000 \times g$ for 10 min. The supernatant from this centrifugation was combined with that of the previous centrifugation and spun at $10\,000 \times g$ for 15 min in a type 42.1 rotor of a Beckman L2-65B ultracentrifuge. The resulting supernatant was set aside. The pellet was resuspended in the sucrose solution and again centrifuged at the same speed. The resulting supernatant was combined with that of the previous step and centrifuged at $120\,000 \times g$ for 60 min.

The resulting supernatant was discarded and the pellet was resuspended in the sucrose solution and used for the binding studies. All of this preparative work was done at 0—4°C. The amount of protein in the membrane preparation was determined by the Lowry method [12] using bovine serum albumin as a standard. The membrane preparation was stored in the sucrose solution at 2°C for no longer than three days before being used.

Binding studies

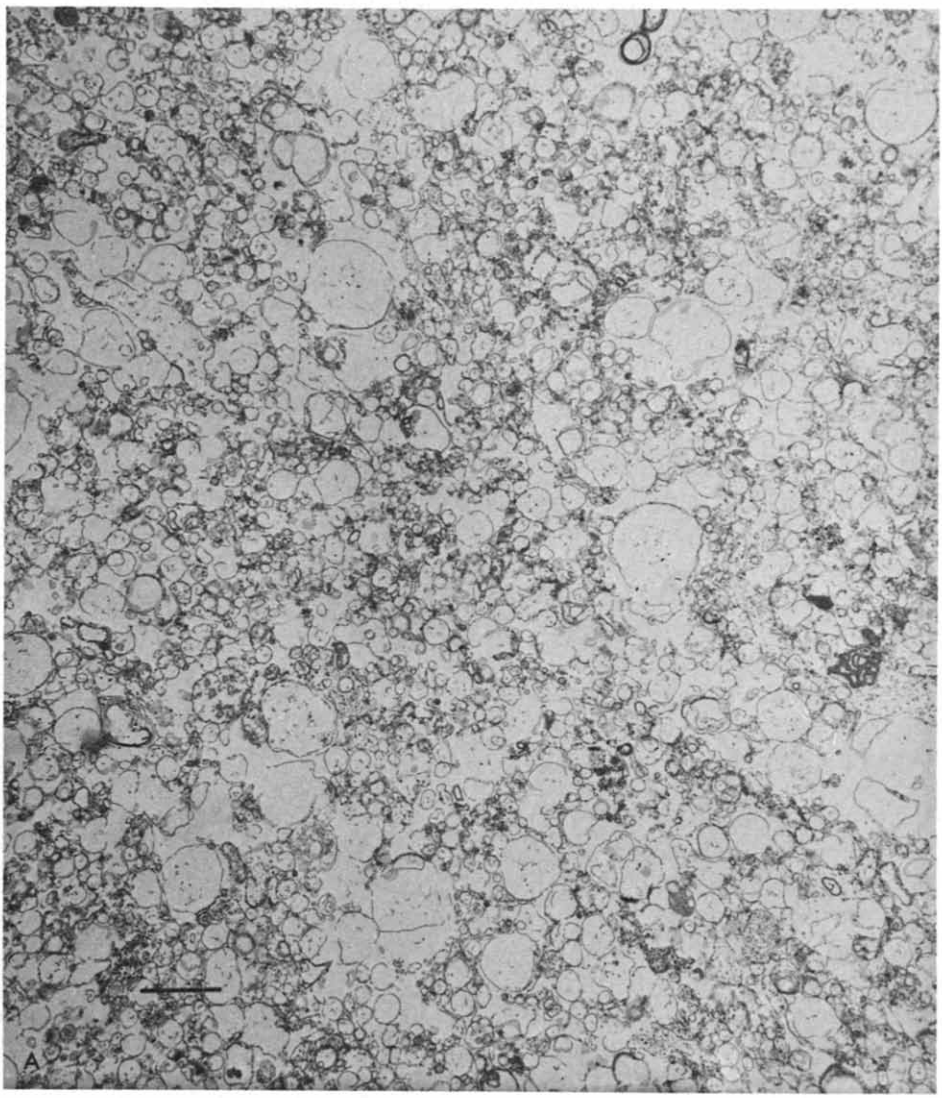
The binding studies were performed as follows. Radioactive calcium was obtained from New England Nuclear at a specific activity of 16 mCi/mg. Enough (non-radioactive) $CaCl_2$ (1 mM) was added to the $^{45}CaCl_2$ in order to lower the specific activity to one-tenth of the original level. The calcium concentration was then adjusted to the desired levels by adding distilled water before being used for the following binding studies. Aliquots of the nerve membrane suspension (25—75 μg protein in 25—50 μl of sucrose solution) were mixed with 1 ml of the desired salt solution and allowed to incubate at room temperature for about 5 min. The radioactive $CaCl_2$ was added and the mixture was allowed to incubate, usually for an additional 9 min. The mixture was then filtered over a Millipore filter (0.45 μm pore size, type HAWP) and the filter was rinsed with 6 ml of a Ca^{2+}-free solution of similar salt composition as the solution used for the incubation. The filter was placed in a scintillation vial and dissolved in 1 ml of methoxyethanol (Eastman, scintillation grade). 10 ml of Aquasol (New England Nuclear) were added to the vial and the radioactivity in the vials was determined using a Beckman LS 130 liquid scintillation counter at an efficiency of 85%.

Electron microscopy

Samples of nerve membrane, prepared as described above, were diluted with either a 450 mM NaCl solution or a 22.5 mM NaCl solution. Both solutions were buffered with 15 mM potassium phosphate at pH 7.3. Following a 15 min incubation, enough glutaraldehyde was added to each solution to bring the final concentration of the fixative to 2%, and the membrane samples were kept at room temperature for another 45 min. The suspension of the membranes were then pelleted by centrifugation at 56 000 $\times g$ (max.) for 45 min. These pellets were post-fixed in osmium tetroxide (1%), dehydrated stepwise in ethanol, and embedded in Epon 812. Thin sections of this material were studied by transmission electron microscopy. The fixation, dehydration, embedding, thin sectioning and electron microscopy were all performed by the Tousimis Research Corporation (Rockville, MD).

Determination of Ca^{2+} using arsenazo III

In order to make sure that the uptake of $^{45}Ca^{2+}$ was not influenced by small amounts of contaminating Ca^{2+} present in the various solutions used, the concentration of Ca^{2+} in the solutions of NaCl and of KCl was determined. The method used for this determination was that described by Gratzer and Beaven [13] in which the absorption changes of the dye arsenazo III (obtained from Sigma) can be used to monitor the concentration of Ca^{2+}. The concentration of Ca^{2+} in a solution of 450 mM NaCl and 15 mM Tris was found to be under



5 μM and that in a solution of 450 mM KCl and 15 mM Tris, under 3.5 μM. These values were close to the detection limit of this method. Since most of the work described in this paper was done at appreciably higher Ca^{2+} concentrations, this amount of contaminating Ca^{2+} was ignored. Furthermore, since the concentration of Ca^{2+} in the NaCl solution was slightly higher than that of the KCl solution, one might expect more Ca^{2+} to be bound in the NaCl solution, if this contaminating calcium were playing a role in the observations made in this work. Since the observed amount of Ca^{2+} bound was higher in KCl than in NaCl soltions, it seems very unlikely that the uptake of Ca^{2+} was influenced by the contaminating calcium.

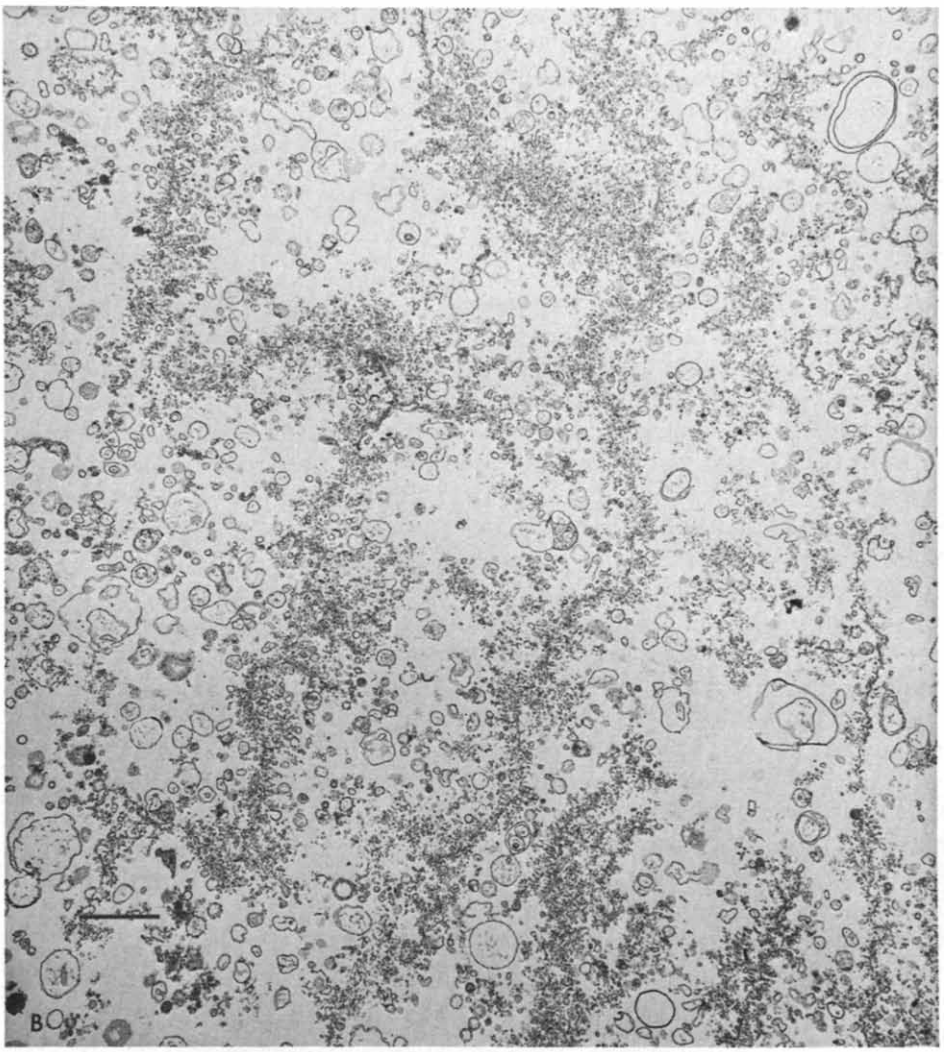


Fig. 1. Electron micrographs of membranes from lobster walking leg nerves. A. Membranes were pelleted from a suspension of membranes in a solution containing 450 mM NaCl, 15 mM potassium phosphate, pH 7.3, before being fixed. B. Membranes were pelleted from a solution containing 22.5 mM NaCl, 15 mM potassium phosphate, pH 7.3. Bar indicates 1 μm.

Determination of the relative membrane potential using merocyanine 540

Merocyanine 540 (obtained from Eastman) was used to compare the relative membrane potential of vesicles suspended in a 450 mM NaCl solution to that of vesicles suspended in a 450 mM KCl solution. The method used was essentially that of Kamino and Inouye [14]. These authors showed that in the presence of this dye, the fluorescence intensity of a suspension of synaptic membrane ghosts increases by incubating them in a monovalent cation salt solution which is known to depolarize an intact nerve. In the present studies, a Perkin-Elmer MPF44A fluorescence spectrophotometer was used. The measurements were

made at room temperature using a concentration of dye of 0.012 mg/ml. The excitation wavelength used was 570 nm and the emission spectrum between 550 and 620 nm was recorded. No differences in the spectrum of a sample of vesicles incubated in a NaCl and a KCl solution was observed, suggesting that the membrane potential across the vesicle membrane was approximately the same in both of these solutions.

Results

Characterization of the membrane vesicles

Fig. 1A shows an electron micrograph of lobster nerve membranes pelleted from a suspension containing 450 mM NaCl. Most of the membranes appear to have formed closed vesicles whose size ranged from over 1 μm in dimater to about 0.1 μm. Some vesicles appeared empty while others contained bits of electron-dense material. No mitochondria were identified in any of the electron micrographs of the membrane pellets thus examined. Fig. 1B is an electron micrograph of the same lobster membrane preparation as in Fig. 1A but these membranes were pelleted from a suspension in a hypotonic solution (22.5 mM NaCl). Although a few vesicles appeared to be intact, most of the electron dense material in this micrograph looked like small (under 0.05 μm) fragments of membrane, clustered together to form ribbons of electron dense material.

Based on the following evidence from the binding of [^{3}H]saxitoxin, the vesicles appeared to be mostly in a right-side-out configuration. Saxitoxin is a potent neurotoxin effective only when applied to the external surface of nerve membranes. It has no electrophysiological effect when applied to the internal surface of intact nerve. This toxin can thus be used as a specific marker for the external surface of nerve membranes. The binding of [^{3}H]saxitoxin to the lobster nerve membrane preparation was studied using a rapid filtration technique (Baumgold, J., unpublished data). It was found that as the ionic strength of the solution was gradually increased (from 25 mM to 600 mM NaCl), the binding of [^{3}H]saxitoxin gradually decreased. Na^+ has been shown to compete with saxitoxin binding in intact nerve and in a solubilized preparation [15]. Since the decrease in the binding of saxitoxin with increasing ionic strength did not exhibit any sigmoidicity that could be attributed to an increase of accessible binding sites, as vesicles opened from a possible inside-out configuration, it was concluded that the majority of the vesicles in the membrane fraction must be in a right-side-out configuration.

Binding studies

Binding studies were performed using the rapid filtration method described above. After filtration, however, it was necessary to rinse the filter with a non-radioactive solution in order to remove the excess unbound Ca^{2+}. This was accomplished by passing a Ca^{2+}-free solution of similar composition as the solution used for incubation through the filter carrying the membrane material. Fig. 2 shows the effect of varying the volume of the solution used to rinse the filters. As can be seen in the figure, the amount of $^{45}Ca^{2+}$ bound to the membranes remained constant when 3—12 ml of solution was used to rinse the filters. This indicated that Ca^{2+} retained after a rinse of at least 3 ml of solution

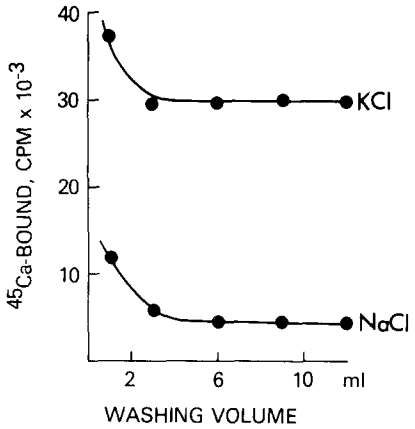


Fig. 2. Effect of rinsing the filters on the amount of Ca^{2+} bound to membrane. Membrane material was incubated with $^{45}Ca^{2+}$ in a solution containing either 450 mM KCl (top) or 450 mM NaCl (bottom) and 15 mM Tris-HCl, pH 8.0. The incubation mixture was filtered and the filter rinsed with the indicated volume of solution containing either 450 mM NaCl (top) or 450 mM KCl (bottom) and 15 mM Tris-HCl, pH 8.0.

was bound fairly tightly. The top curve in Fig. 2 was obtained from aliquots of membrane material suspended in a solution containing 450 mM KCl, 15 mM Tris-HCl, pH 8.0, and 20 μM $^{45}CaCl_2$. The lower curve was obtained from membrane material containing the same amount of protein suspended in a solution containing 450 mM NaCl instead of the KCl. The amount of $^{45}Ca^{2+}$ bound to the membranes was much greater in the KCl solution than in the NaCl solution.

The reason for rinsing the filters was to reduce the rather high background due to the adsorbtion of $^{45}Ca^{2+}$ to the filters. In order to make sure that this rinsing procedure did not remove bound $^{45}Ca^{2+}$, the experiments in Fig. 3 (top) were repeated in the absence of any rinsing procedure. Both the shape and the absolute values of the curves depicted in Fig. 3 (top) could be fairly well reproduced without rinsing the filters, although in the absence of rinsing, the noise in the measurements was considerably increased.

Fig. 3 (top) shows the amount of $^{45}Ca^{2+}$ bound to the membrane material as a function of the calcium concentration. The free Ca^{2+} concentration in the incubating solution ranged from 1 to 100 μM. In some experiments, the calcium concentration was raised to several hundred μM (data not shown) resulting in a continued increase in the amount of bound $^{45}Ca^{2+}$.

In order to determine why so much more Ca^{2+} was bound to membranes that were incubated in a 450 mM KCl solution compared with membranes that were incubated in a 450 mM NaCl solution, a similar binding experiment was performed by incubating the membranes in 22.5 mM salt solutions (Fig. 3, bottom). As seen in the figure, the use of low ionic strength salt solutions resulted in a very small difference in the amount of $^{45}Ca^{2+}$ bound to the membranes in the NaCl vs. KCl containing solutions.

The ionic strength effect illustrated in Fig. 3 can be explained as follows. At high ionic strength, the membranes form vesicles (see Fig. 1) which are, presumably, capable both of accumulating Ca^{2+} into the vesicle and of binding Ca^{2+} to anionic sites on the membrane. At low ionic strength, the membrane

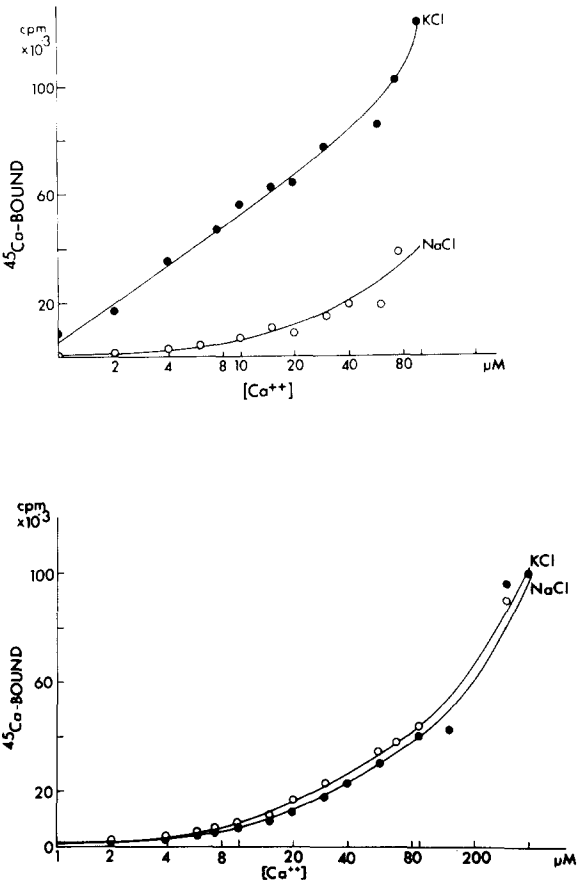


Fig. 3. Relation between the amount of Ca^{2+} bound to membrane material and the calcium ion concentration in the incubation medium. The solution used for the incubation contained 450 mM NaCl or KCl in the top panel and 22.5 mM NaCl or KCl in the bottom panel.

material is predominantly in the form of fragments which are incapable of Ca^{2+} accumulation. Since at low ionic strength the membrane fragments bind $^{45}Ca^{2+}$ almost as well in NaCl as in KCl, the two monovalent cations of these salts must compete to the same extent for the $^{45}Ca^{2+}$-binding sites. On the assumption that these sites remain incapable of distinguishing between the monovalent cations at higher ionic strengths (an assumption for which some experimental support can be found in Fig. 6), the difference between the amount of $^{45}Ca^{2+}$ bound to the membrane fragments incubated in a KCl solution and that bound in a NaCl solution must then be due to differences in $^{45}Ca^{2+}$ accumulation rather than $^{45}Ca^{2+}$ binding.

From the Scatchard plots of the binding data, shown in Figs. 4 and 5, the existence of two distinct binding components can be recognized. When the binding studies were performed in a high ionic strength (450 mM) solution, the dissociation constant for the higher affinity sites was not dependent on which salt (NaCl or KCl) was used in the incubating solution: the dissociation constant was 16 ± 10 μM (n = 2) in 450 mM NaCl and 13 ± 2 μM (n = 3) in

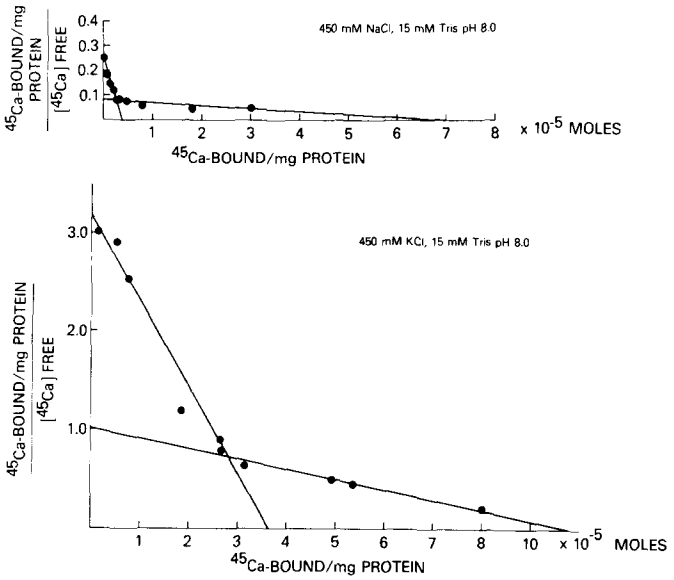


Fig. 4. Scatchard plots of the binding data. The solutions used for the incubation are as indicated in the figures.

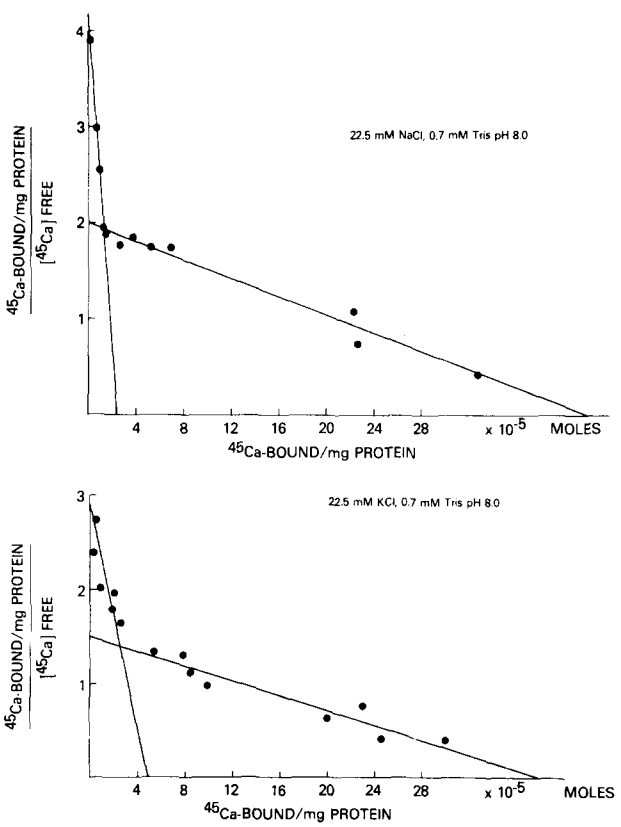


Fig. 5. Scatchard plots of the binding data. The solutions used for the incubation are as indicated in the figure.

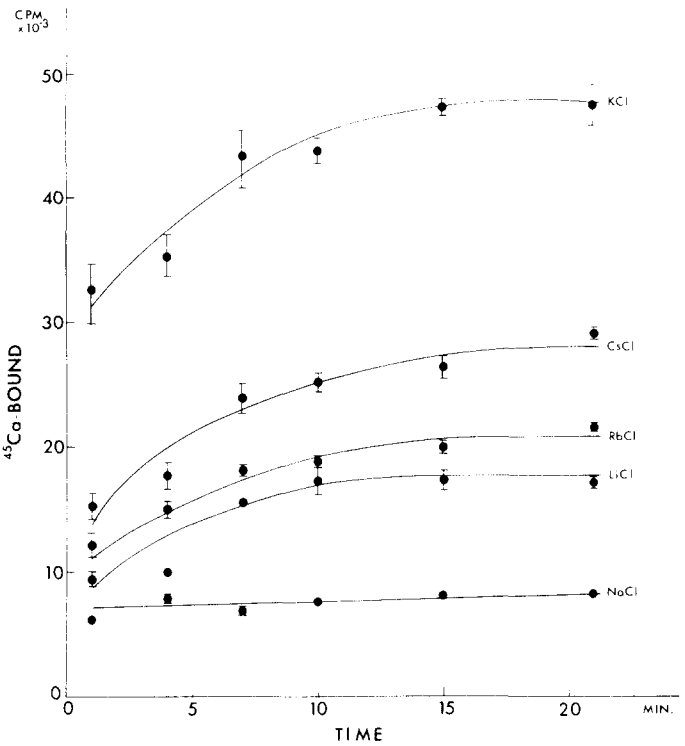


Fig. 6. Dependence of the amount of Ca^{2+} bound to membrane material on the duration of incubation. The membrane material was incubated in 450 mM of the indicated salt solution and 15 mM Tris-HCl, pH 8.0. At zero time, $^{45}Ca^{2+}$ was added. The mixtures were filtered at the indicated times. Each point is the mean of three determinations and the error bars represent ±1 standard deviation about the mean.

450 mM KCl. The binding capacity of these higher affinity sites, however, was strongly dependent on what kind of salt was used in the incubating solution: the higher affinity site had a capacity of 32 ± 9 μmol (n = 3) of Ca^{2+} bound/mg protein in the KCl containing solution but only 3.5 ± 5 μmol (n = 2) of Ca^{2+} bound/mg protein in the NaCl containing solution (see Table I). The dissociation constant for the lower affinity sites was 0.17 ± 0.04 mM (n = 3) in KCl and 0.5 ± 0.28 mM (n = 3) in NaCl. These sites had Ca^{2+} binding capacities 89 ± 14 μmol (n = 3)/mg protein and 46 ± 20 μmol (n = 3)/mg protein, respectively.

At low ionic strength (Fig. 5) the dissociation constants of the higher affinity site were similar (within a factor of close to 2) to those found at high ionic strength (K_d = 5.7 μM in NaCl and 17 μM in KCl). The Ca^{2+} binding capacity of these sites was 26 μmol/mg protein in NaCl and 46 μmol/mg protein in KCl. The lower affinity sites had capacities of 0.42 mmol Ca^{2+}/mg protein in NaCl and 0.38 mmol Ca^{2+}/mg protein in KCl, and dissociation constants of 0.21 mM and 0.25 mM, respectively. The results of these determinations are summarized in Tables I and Table II.

TABLE I

DISSOCIATION CONSTANTS AND BINDING CAPACITIES FOR THE BINDING OF $^{45}Ca^{2+}$ TO MEMBRANES INCUBATED IN HIGH IONIC STRENGTH SOLUTIONS

	450 mM NaCl		450 mM KCl	
High affinity				
Dissociation constant	16 ± 1 μM	(n = 2)	13 ± 2 μM	(n = 3)
Binding capacity *	3.5 ± 5	(n = 2)	32 ± 9	(n = 3)
Low affinity site				
Dissociation constant	0.54 ± 0.28 mM	(n = 3)	0.17 ± 0.04 mM	(n = 3)
Binding capacity *	46 ± 20	(n = 3)	89 ± 14	(n = 3)

* Expressed in micromoles of bound Ca^{2+} per mg protein.

Kinetic studies

The time course of Ca^{2+} binding is shown in Fig. 6. The time course was found to be strongly dependent upon the monovalent cation species of the salt used in the suspending solution. In the NaCl containing solution $^{45}Ca^{2+}$ bound rapidly to the membrane, achieving apparent equilibrium within 1 min and remaining constant for the following 20 min. In the KCl containing solution, however, two rate constants were distinguishable: a high rate which brought the system to apparent equilibrium within the first minute, and a low rate which required about 13 min to achieve apparent equilibrium. Tests with three other salts indicated the existence of both the fast and the slow processes of Ca^{2+} binding as shown in Fig. 6. Since apparent equilibration was so rapid with the fast process, it was not possible to accurately study its rate using the filtration technique. However, it is clear that, if the curves for the CsCl, RbCl, LiCl and NaCl solutions in Fig. 6 are extrapolated to zero time, they do not intersect the ordinate at zero, but rather in the vicinity of $7 \cdot 10^3$ cpm (due to the rather large experimental error in the KCl series, little can be concluded from that curve). This amount of $^{45}Ca^{2+}$ bound must then be due to the sorption of $^{45}Ca^{2+}$ to superficially located membrane sites. Since the amount of Ca^{2+} bound via this fast process is similar for a least four out of the five salts tested (and quite possibly for KCl as well), the interpretation of the results suggests that the difference in the species of monovalent cations in the incubating solution

TABLE II

DISSOCIATION CONSTANTS AND BINDING CAPACITIES FOR THE BINDING OF $^{45}Ca^{2+}$ TO MEMBRANES INCUBATED IN LOW IONIC STRENGTH SOLUTIONS

	High affinity site		Low affinity site	
	Dissociation constant (μM)	Binding capacity *	Dissociation constant (mM)	Binding capacity *
22.5 mM NaCl	5.7	26	0.21	420
22.5 mM KCl	17	46	0.25	380

* Expressed in μmol of bound Ca^{2+} per mg protein.

does not significantly influence the amount of $^{45}Ca^{2+}$ adsorbed to the membranes.

Effect of monovalent cations

Fig. 7 shows the effect of a series of monovalent salts on the amount of $^{45}Ca^{2+}$ bound to the membranes. The amount of $^{45}Ca^{2+}$ bound was highest in a KCl solution, lowest in a NaCl solution and followed the order KCl > CsCl > RbCl > LiCl > NaCl. This order is different from that in which monovalent cations depolarize intact nerve.

Ionic strength effects

The effects of varying the ionic strength of the incubating solution on the binding of $^{45}Ca^{2+}$ is shown in Fig. 8. When the concentration of the NaCl in the solution was increased, the amount of bound $^{45}Ca^{2+}$ gradually decreased (Fig. 8, top). This is consistent with the notion that in a NaCl containing solution, Ca^{2+} binds only to the accessible membranes sites, without crossing the membrane. As the NaCl concentration was raised, sodium competed more effectively for the Ca^{2+} binding sites, resulting in the observed decrease in bound $^{45}Ca^{2+}$. When KCl was used in the suspending solution, however, the amount of $^{45}Ca^{2+}$ increased, as the concentration of KCl was raised from 100 mM, reaching a maximum at around 200 mM KCl, before gradually declining. This initial increase in the bound Ca^{2+} may reflect an increase in the population of closed vesicles capable of accumulating calcium in their interiors. As the number of closed vesicles increased, the amount of Ca^{2+} which accumulated in those vesicles was expected to increase, resulting in the observed change shown in Fig. 8. The gradual decline observed when the KCl con-

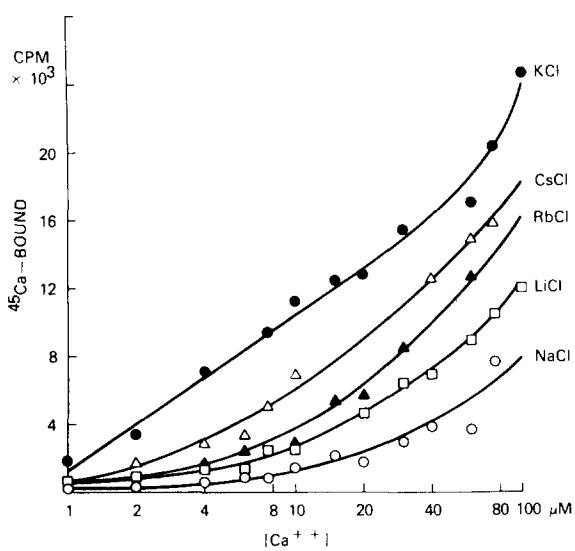


Fig. 7. Effect of various monovalent cations on the amount of Ca^{2+} taken up by the membrane material. In each case, the anion was chloride and a 450 mM solution of the salt was used, along with 15 mM Tris-HCl, pH 8.0.

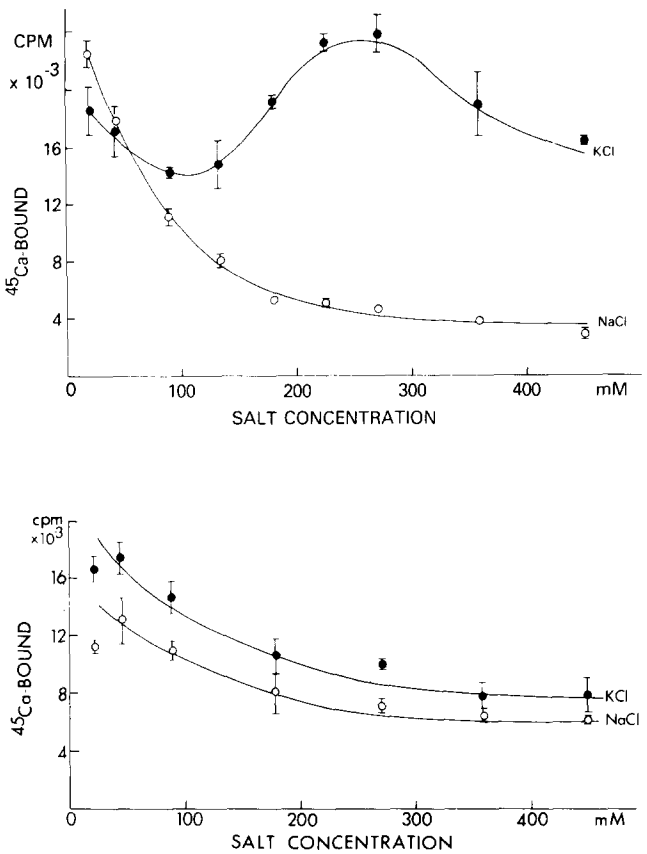


Fig. 8. Dependence of the amount of Ca^{2+} bound to membrane material on the salt concentration. The concentration of KCl or NaCl in the incubation medium was varied as indicated. The membrane samples were incubated in the solutions for 5 min before the $^{45}Ca^{2+}$ was added and for 9 min after the addition of $^{45}Ca^{2+}$. The concentration of $^{45}Ca^{2+}$ in the incubating solution was 40 μM. In the lower panel, the membranes were subjected to a hypotonic shock by rinsing them in distilled water. Each point is the mean of three determinations and the error bars represent ±1 standard deviation about the mean. The bars were omitted when the standard deviation was smaller than the size of the symbols.

centration was raised beyond 200 mM, can be explained by assuming that the number of closed vesicles did not increase beyond 200 mM KCl and that K^+ competes more and more effectively for the Ca^{2+}-binding sites on the membrane surface. The initial decrease in the amount of bound $^{45}Ca^{2+}$ seen between 22.5 and 100 mM may be due to increasing competition of K^+ for the superficial membrane sites. The lower portion of the figure shows the results obtained from membrane fragments which were once subjected to hypotonic shock by rinsing them in distilled water, before the $^{45}Ca^{2+}$ binding was determined. In this case, the amount of Ca^{2+} bound decreased gradually with increasing concentration of either NaCl or KCl. Thus, after being washed with distilled water, the membranes became incapable of accumulating Ca^{2+} into the vesicles.

Further evidence that the accumulation of Ca^{2+} in the vesicles is dependent

upon the monovalent cation species in the solution used for suspending the vesicles is shown in Fig. 9. If the difference in amount of $^{45}Ca^{2+}$ bound to membranes incubated in various monovalent cation salt solutions was due to the differing ability of the cations to compete for the Ca^{2+} binding sites on the membrane, then by gradually increasing the ratio of KCl to NaCl in the suspending solution, we might expect a steadily increasing amount of bound $^{45}Ca^{2+}$. As seen in Fig. 9, however, the amount of bound Ca^{2+} does not increase steadily but rather first increases very gradually and then very sharply as the ratio of KCl to NaCl in the suspending solution is increased. This suggests that the difference in the amount of Ca^{2+} bound in KCl versus NaCl solutions cannot be due to the ability of Na^+ to compete for the Ca^{2+} sites better than K^+. Instead, the data in Fig. 9 implie that the ratio of NaCl to KCl plays an important role in determining whether Ca^{2+} will be allowed to accumulate inside the vesicle. It is interesting to note that the addition of as little as 50 mM NaCl to a 400 mM KCl solution can be enough to considerably reduce the Ca^{2+} accumulation of the vesicles. This rather sharp transition is suggestive of a two-state system, in which Ca^{2+} is allowed to accumulate readily into the vesicles in one state, and accumulates so slowly in the other state that almost no Ca^{2+} accumulated during the incubation periods used in these experiments.

Release of Ca^{2+} by hypotonic shock

Membrane vesicles were loaded with $^{45}Ca^{2+}$ by incubation in the presence of 100 μM $^{45}Ca^{2+}$ and 450 mM KCl. They were then filtered and rinsed with the 450 mM KCl solution. The vesicles were then subjected to a hypotonic shock by rinsing them with two 1-ml aliquots of a solution containing 22.5 mM KCl. The solution used for these final two rinses was collected and the amount of $^{45}Ca^{2+}$ determined. Table III shows the results of these experiments. The

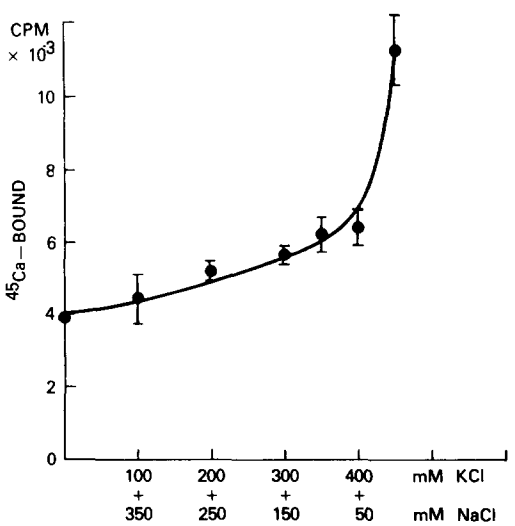


Fig. 9. Effect of varying the Na^+ : K^+ concentration ratio on the amount of bound Ca^{2+}. The composition of the incubation solution was varied as indicated.

TABLE III

HYPOTONIC RELEASE OF $^{45}Ca^{2+}$ FROM MEMBRANE VESICLES

Washing solution	$^{45}Ca^{2+}$ in first wash	$^{45}Ca^{2+}$ in second wash
450 mM NaCl	39 333	28 384
22.5 mM NaCl	41 420	8 186
450 Mm KCl	23 877	14 921
22.5 mM KCl	84 365	28 060

amount of $^{45}Ca^{2+}$ in the hypotonic solution used to rinse the vesicles was much larger than the amount in a control sample in which 450 mM KCl was used for the final two rinses, instead of the hypotonic solution. When NaCl was used no increase in $^{45}Ca^{2+}$ was detected in the hypotonic rinsing solution. These experiments suggest that as the vesicles containing $^{45}Ca^{2+}$ were exposed to the hypotonic rinsing solution, they opened, thereby releasing a portion of their $^{45}Ca^{2+}$ that had been contained within the vesicle.

Pharmacological studies

The following pharmacological agents were tested in solutions containing 450 mM of either NaCl or of KCl. Pre-incubation or co-incubation of the membranes with metabolic inhibitors such as azide (1 mM) or with the ATPase inhibitor, ouabain (1 mM), had no effect on the amount of Ca^{2+} bound, suggesting that the Ca^{2+} binding was not dependent on metabolic energy. Furthermore, the addition of 3 mM $MgCl_2$, 3 mM ATP or 3 mM oxalate, added either separately or together, failed to increase the amount of bound Ca^{2+}.

Neither tetrodotoxin ($3 \cdot 10^{-6}$ M) nor veratridine ($2 \cdot 10^{-5}$ M) had any effect in altering the amount of calcium-45 bound to the membranes.

Once bound, very little calcium could be desorbed by adding an excess (2 mM) of ethylenediamine tetraacetic acid (EDTA) to the solution. In the NaCl containing solution, 4.2% of the bound $^{45}Ca^{2+}$ was desorbed, and in the KCl solution, 11.1% was desorbed. The small amount of bound Ca^{2+} that was removed with EDTA suggests that Ca^{2+} binds to sites not readily accessible to EDTA.

Discussion

The results presented in this study show two distinct components for the binding of Ca^{2+} to lobster nerve membranes (Fig. 4). The higher affinity component is sensitive to the monovalent cation species used in the incubating solution and, from the ionic strength data (Fig. 8), can be said to be due to the accumulation of Ca^{2+} in the vesicles. This Ca^{2+} accumulation occurs readily when the membranes are incubated in a KCl containing solution but occurs so slowly in a NaCl containing solution that no accumulation was detected in these experiments. The lower affinity component appears to be insensitive to the salt species used in the incubating solution and may be due to the adsorption of Ca^{2+} to anionic sites on the membrane. The rate of absorption of Ca^{2+}

from these sites must be slow since Ca^{2+} is not displaced by extensive rinsing of the membranes with a Ca^{2+}-free solution. Thus, although the monovalent cation species in the incubation medium has a dramatic effect on the accumulation of Ca^{2+} into the vesicles, it has little effect on the binding of Ca^{2+} to the membrane sites.

Previous investigations have shown in whole nerve that the uptake of Ca^{2+} can occur either in response to depolarization [8] or to manipulation of the external Na^+ concentration [9,10]. Thus, it has been shown in crab nerve [9], in squid giant axon [10] and in isolated synaptic terminals [16] that by substituting Li^+ or dextrose for Na^+, the influx of Ca^{2+} could be increased, suggesting an antagonism between the presence of external Na^+ and Ca^{2+} influx. This manipulation of the external ionic composition did not cause depolarization. In a similar fashion, by replacing external NaCl with LiCl in the present studies, the uptake of $^{45}Ca^{2+}$ into membrane vesicles could be enhanced. This strongly suggests that the uptake of $^{45}Ca^{2+}$ seen when NaCl was replaced with any of the other monovalent cations tested was due to a mechanism which is inhibited by Na^+, as previously described for intact nerve. Furthermore, the following three observations provide evidence that this enhanced Ca^{2+} accumulation seen in the present studies was not in response to depolarization of the vesicle membrane. (1) The order in which the monovalent cations stimulate Ca^{2+} uptake in the present experiments is very different from the order in which they depolarize intact nerve. (2) The membrane potential across the vesicle membrane was found to be approximately the same whether the vesicle was incubated in NaCl or in KCl. (3) Veratridine, an agent capable of depolarizing intact nerve, failed to stimulate Ca^{2+} accumulation in the present experiments.

Thus homogenized lobster nerve membrane material provides a useful preparation on which to study the binding of Ca^{2+}, since by manipulating the tonicity of the bathing medium, it becomes possible to distinguish between the binding of Ca^{2+} to anionic membrane sites and the accumulation of Ca^{2+} into vesicles.

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References

1 Tasaki, I. (1968) Nerve Excitation, A Macromolecular Approach, Charles C. Thomas, Springfield, IL
2 Koketsu, K. (1969) in Neurosciences Research (Ehrenpreis, S. and Solnitzky, O.C., eds.), Academic Press
3 Kamino, K., Uyesaka, N. and Inouye, A. (1974) J. Membr. Biol. 17, 13—26
4 Lieberman, E.M., Palmer, R.F. and Collins, G.H. (1967) Exp. Cell Res. 46, 412—418
5 Abood, L.G. (1969) Neurosciences Research (Ehrenpreis, S. and Solnitsky, O.C., eds.), Academic Press
6 Abood, L.G. and Hoss, W. (1974) Biochim. Biophys. Acta 332, 85—96
7 Baker, P.F. and McNaughton, P.A. (1978) J. Physiol. 276, 127—150
8 Baker, P.F. and Glitsch, H.G. (1975) Philos. Trans. R. Soc. Lond. B 270, 389—409

9 Baker, P.F. and Blaustein, M.P. (1968) Biochim. Biophys. Acta 150, 167—170
10 Baker, P.F., Blaustein, M.P., Hodgkin, A.L. and Steinhardt, R.A. (1969) J. Physiol. 200, 431—458
11 Denburg, J.L. (1972) Biochim. Biophys. Acta 282, 453—458
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
13 Gratzer, W.B. and Beaven, G.H. (1977) Anal. Biochem. 81, 118—129
14 Kamino, K. and Inouye, A. (1978) Jap. J. Physiol. 28, 225—237
15 Henderson, R., Ritchie, J.M. and Strichartz, G.R. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3936—3940
16 Blaustein, M.P. and Wiesmann, W.P. (1970) Proc. Natl. Acad. Sci. U.S. 66, 664—671