BBA 75055

PHOSPHOLIPIDS AS ION EXCHANGERS: IMPLICATIONS FOR A POSSIBLE ROLE IN BIOLOGICAL MEMBRANE EXCITABILITY AND ANESTHESIA

M. P. BLAUSTEIN*
Biophysics Division, Naval Medical Research Institute, Bethesda, Md. (U.S.A.)

(Received March 10th, 1967)

SUMMARY

1. Distribution ratios between a chloroform–methanol phase (with and without phosphatidyl ethanolamine or phosphatidyl serine) and an aqueous (Ringer) phase were measured for a number of ions and drugs. Net increase of test agent in the chloroform phase in the presence of phospholipid was taken as a measure of binding to the phospholipid.

2. Anionic and cationic anesthetic agents (such as barbiturates and procaine-like drugs) bind to phospholipids. Anionic drugs increase the binding of calcium to phospholipids; cationic drugs decrease calcium binding.

3. Various polyvalent cations decrease the binding of calcium to phosphatidyl serine. The relative ability of these ions to decrease calcium binding is presumably a function of the relative binding affinity of the ions for phosphatidyl serine.

4. The binding properties of the ions and drugs to phosphatidyl serine can be correlated with their effects on peripheral nerve.

5. The properties of this ion-exchanger system provide a useful model for the interpretation of polyvalent cation and anesthetic effects on the nerve membrane.

INTRODUCTION

Interest in the contribution of cell lipids to biological membrane permeability processes dates back to OVERTON's[1,2] demonstration of a correlation between cell permeability and the oil–water partition coefficient of various substances. In 1925, GORTER AND GRENDEL[3] showed that the total lipid extracted from an erythrocyte, when spread in a monolayer, was equal to twice the surface area of the erythrocyte. They therefore proposed that the plasma membrane was composed of a bimolecular lipid layer. Their theory was revised by DAVSON AND DANIELLI[4] who suggested that

Abbreviation: PHMB, *p*-hydroxymercuribenzoate (formerly called PCMB, p-Cl mercuribenzoate, according to literature from the Sigma Chemical Company, St. Louis).

* Present address: Physiological Laboratories, University of Cambridge, Cambridge (England).

the plasma membrane was a very thin lipid layer coated with protein on both its inner and outer surfaces.

The Davson–Danielli model has received considerable experimental support from analytical biochemistry, electron microscopy and X-ray diffraction studies (refs. 5–7, but see ref. 8). Although a significant proportion of the lipid of many, if not all, cell membranes is phospholipid[8,9], little is known about the precise role which the lipids and particularly the phospholipids may play in physiological processes. It is known that various cations, particularly polyvalent cations, will bind to phospholipids[10–15], and GOLDMAN[16] has suggested that the polar heads of membrane phospholipids may contain the ion-exchange sites controlling permeability to Na^+ and K^+ during neuronal excitation. These observations and ideas, *plus* the demonstration of competitive interaction between Ca^{2+} and local anesthetics in phospholipid systems[10,12,17] as well as in peripheral nerve (reviewed in refs. 18, 19) suggest that a detailed study of the ion and drug binding characteristics of various phospholipids might provide useful information to correlate with data from peripheral nerve studies. The present study was undertaken toward this end. Preliminary reports of some of the data have been published[10,20].

METHODS

The techniques employed for these experiments were essentially the same as those used by FEINSTEIN[12] for the measurement of calcium binding to phospholipids. 1.0 ml of aqueous solution containing 116 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$ and 2.0 mM Tris (at pH 7.4, except as noted below) was added to 2.0 ml of a 2:1 mixture of chloroform–methanol (v/v) containing 1 mg of phospholipid per ml. $^{45}Ca^{2+}$ was initially present in the aqueous solution at a specific activity of about 1 μC/mmole of $CaCl_2$. The 3.0-ml mixture was shaken for 10 min and then centrifuged for 5 min at about 1000–2000 $\times$ *g* to separate the chloroform and aqueous phases. Both phases were assayed for radioactivity using either a gas-flow or liquid-scintillation counter. Various modifications of this basic procedure, such as the addition of ions or drugs or the use of ^{14}C-labeled drugs, will be described below. The phospholipids used in these experiments were: Grade II synthetic L-α-cephalin (β,γ-dipalmitoyl-L-α-glyceryl phosphoryl-ethanolamine) and Grade II, phosphatidyl-L-serine (Fraction III of Folch), both of which were obtained from the Sigma Chemical Company, St. Louis.

Ascending chromatography on silicic acid-impregnated paper with a di-isobutyl ketone–acetic acid–water (40:25:5) solvent system[21] was used to check the phospholipids. The phosphatidyl ethanolamine was observed to have a single spot with an R_F value of 0.68. Chromatography of the phosphatidyl serine yielded only 2 spots, one of which was small, brownish in color, and remained at the origin; while the second, much larger spot, traveled with an R_F of 0.53. It thus seems unlikely that lipids other than phosphatidyl serine may have been present in high enough concentration to account for a significant proportion of observed ion binding. In the experiments to be described below, the phosphatidyl serine was used without removing any of the impurities.

In the absence of phospholipid, about 1% or less of the $^{45}Ca^{2+}$ was taken into the chloroform phase. In control experiments with ^{14}C-labeled phosphatidyl ethanolamine (obtained from Applied Science Laboratories), more than 98% of this

lipid remained in the chloroform phase during the course of an experiment. BREYER AND QUADBECK[11] found a similar distribution for phosphatidyl serine. It therefore seems likely that virtually all of the lipid remains in the chloroform phase and that when Ca^{2+} is taken into the chloroform phase, it is the result of binding to the lipid.

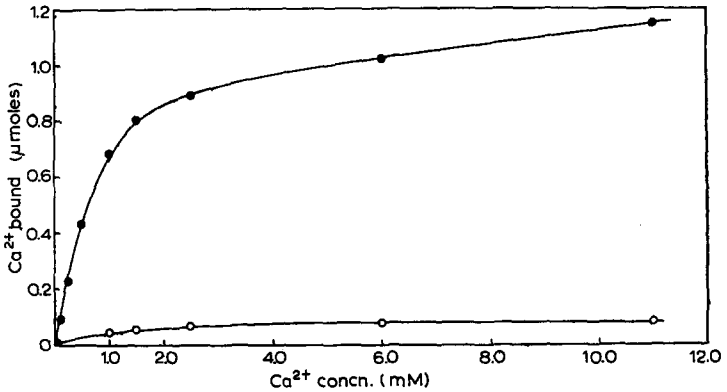

Fig. 1. Effect of Ca^{2+} concentration on calcium binding to phosphatidyl serine (●—●) and phosphatidyl ethanolamine (○—○). Ca^{2+} concentration given in terms of μmoles per ml of original aqueous solution. Ordinate is in terms of total number of μmoles of Ca^{2+} bound to 2 mg of phospholipid.

RESULTS

1. *Ca^{2+} binding to phospholipids: influence of lipid and Ca^{2+} concentration, pH, and organic solvent*

The effect of Ca^{2+} concentration on calcium binding by phosphatidyl serine and phosphatidyl ethanolamine is illustrated in Fig. 1. The curves suggest that the equivalent binding capacity of the lipid preparations used is about 0.5 μmole of Ca^{2+} per mg of crude phosphatidyl serine and about 0.05 μmole of Ca^{2+} per mg of phosphatidyl ethanolamine.

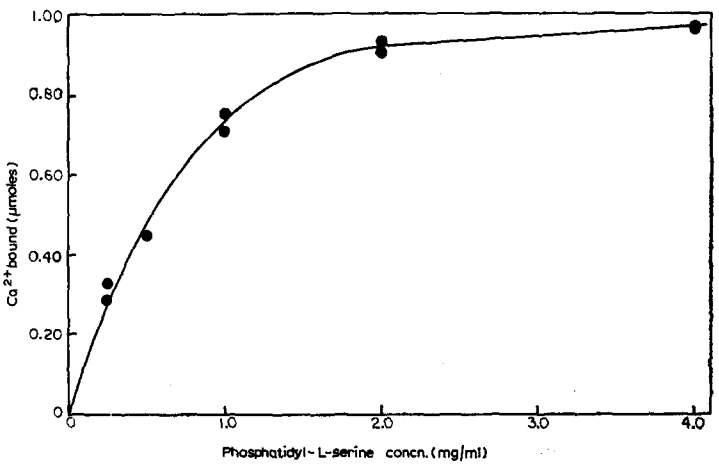

Fig. 2. Effect of phosphatidyl serine concentration on the binding of Ca^{2+} to phospholipid. Lipid concentration is given in terms of mg of lipid per ml of original 2:1 chloroform–methanol solution.

The relationship of phosphatidyl serine concentration to Ca^{2+} binding for a constant Ca^{2+} concentration in the aqueous phase, is shown in Fig. 2. The curves in Figs. 1 and 2 demonstrate that at low Ca^{2+} or low phosphatidyl serine concentrations there is an approximate binding ratio of 0.5 μmole of Ca^{2+} per mg of lipid (since 1.0 ml of the original aqueous solution is added to 2.0 ml of the solution of lipid in chloroform). Furthermore, when 1.0 ml of aqueous solution, containing 1.0 μmole of $CaCl_2$, is mixed with 2.0 ml of chloroform–methanol containing 1 mg/ml phosphatidyl serine, about 0.75 μmole of Ca^{2+} is bound to the phosphatidyl serine, so that the lipid is about three-quarters saturated.

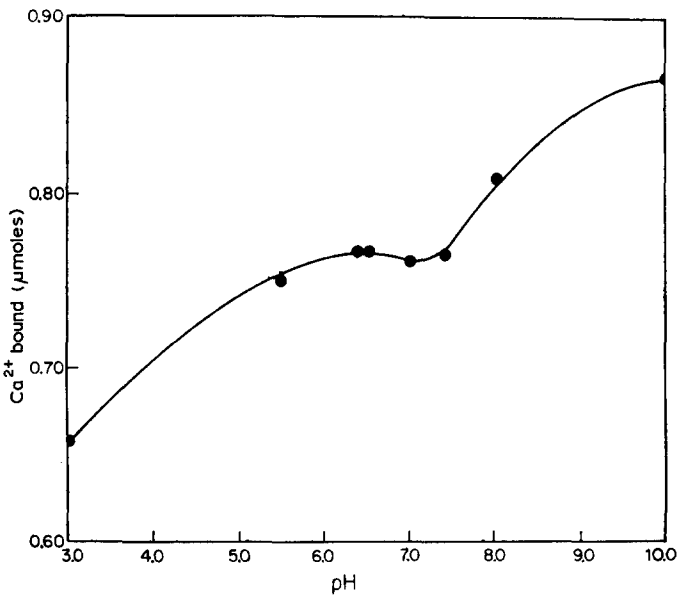

Fig. 3. Effect of pH on the binding of Ca^{2+} to phosphatidyl serine.

Fig. 3 demonstrates the effect of pH on Ca^{2+} binding to phosphatidyl serine. The shape of the curve is qualitatively similar to that obtained by ROJAS AND TOBIAS[15] with a phosphatidyl serine monolayer. As these investigators pointed out, this pH curve may be correlated with GARVIN AND KARNOVSKY'S[22,23] finding that the amino group of phosphatidyl serine has an apparent pK_a of about 10.3, and the carboxyl group has a pK_a of 4.6, while the pK_a of the phosphate group is much less than 4. If these observations apply to the data of Fig. 3, we would expect all three ionizable groups in the phospholipid to be ionized in the plateau region between pH 6 and pH 8.

The choice of organic solvents plays a significant role in determining the amount of Ca^{2+} binding; thus, in Fig. 4, Ca^{2+} binding to phosphatidyl serine is maximal when the chloroform–methanol ratio is about 3 or 4 to 1. A large increase or decrease in this ratio markedly reduces the observed Ca^{2+} binding. Since the distribution of lipid between aqueous and chloroform phases, as a function of the chloroform–methanol ratio, was not determined, the data of Fig. 4 have not been corrected for this possible source of error.

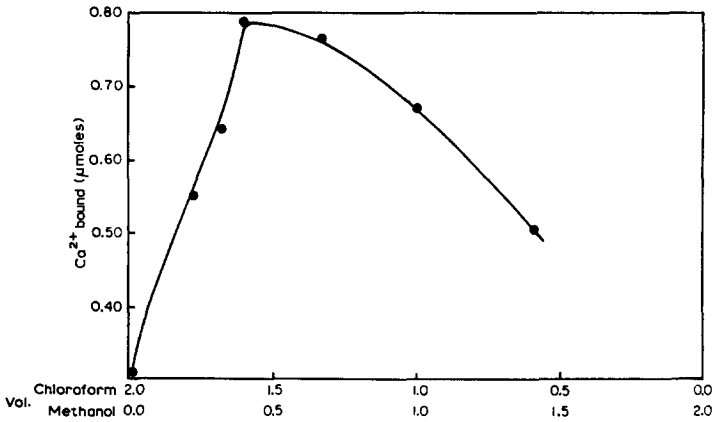

Fig. 4. Effect of the chloroform–methanol ratio on the binding of Ca^{2+} to phosphatidyl serine.

Several other solvent systems were also tested. Ca^{2+} binding to phosphatidyl serine was slightly decreased when 2:1 chloroform–ethanol was used in place of chloroform–methanol. *n*-Propanol and *n*-butanol were still poorer substitutes for methanol in this respect. There was 50% less Ca^{2+} bound when 2:1 chloroform–dioxane was used in place of 2:1 chloroform–methanol.

Benzene and toluene, like chloroform, were poor solvents for the demonstration of Ca^{2+} binding to phosphatidyl serine and phosphatidyl ethanolamine, in the absence of a lower alcohol. Ratios of 2:1 benzene–methanol or toluene–methanol were about as effective as 2:1 chloroform–methanol, in terms of Ca^{2+} binding. Although 2:1 chloroform–methanol was used as the lipid solvent for all of the studies to be described below, preliminary drug and ion experiments employing these other organic solvent systems yielded results similar to those obtained with the use of chloroform–methanol.

2. Effect of other polyvalent cations on Ca^{2+} binding to phospholipids

The relative ability of a number of polyvalent cations to inhibit the binding of $^{45}Ca^{2+}$ to phospholipids was employed as an indirect measure of the relative binding strength of these other cations to the phospholipids. For these experiments, the concentration of test cation in the original aqueous phase (c') required to inhibit $^{45}Ca^{2+}$ binding to the same degree as 0.5 mM unlabeled Ca^{2+} (c) was compared for the various cations. The results, for binding to phosphatidyl serine, computed as c/c', are presented in the second column of Table I. In all of these experiments, several concentrations of each of the test ions were tried and in most instances, c' was calculated from a linear interpolation between values slightly greater than and slightly less than c'. The relative differences between cations were much less marked when phosphatidyl ethanolamine was the lipid used.

The data for Ca^{2+} and Mg^{2+} may also be expressed in terms of HELFFERICH's[25] "separation factor",

$$\frac{[Ca]_o/[Mg]_o}{[Ca]_a/[Mg]_a}$$

where the subscripts "a" and "o" refer to the equilibrium concentrations of cation in

the organic and aqueous phases, respectively. Since 0.80 μmole of Ca^{2+} is bound to phosphatidyl serine when 1.5 μmoles are present initially (Fig. 1), we assume the same total amount of cation bound to phospholipid when the aqueous phase initially contains 1.0 μmole of Ca^{2+} *plus* 1.25 (*i.e.*, 0.5/0.4, from Table I) μmoles of Mg^{2+}. Under the latter conditions, 0.53 μmole of Ca^{2+} is bound, giving a separation factor of 4.2, in reasonable agreement with the value of 5 obtained by BREYER AND QUADBECK[11].

Inhibition of [^{14}C]procaine binding (see below for methods used to calculate this quantity) to phosphatidyl serine by several cations was also studied. The relative concentrations of Ca^{2+} (c) and of another test ion (c') required to reduce procaine binding by 50% were also compared (c/c'); these data are given in the third column of Table I.

TABLE I

EFFECT OF CATIONS ON BINDING OF Ca^{2+} AND OF PROCAINE TO PHOSPHATIDYL SERINE

Cation (0.5 mM)	*Relative inhibition of binding of*		*Relative effectiveness as Ca^{2+} replacement in lobster axon**
	1 mM Ca^{2+}	*2 mM [^{14}C]-procaine*	
Mg^{2+}	0.4	0.6	0.2
Ca^{2+}	(1.0)	1.0	1.0
Cd^{2+}	1.2	—	3–4
Sr^{2+}	1.3	1.1	—
Co^{2+}	1.4	—	4–5
Mn^{2+}	1.5	—	4–5
Ba^{2+}	1.9	1.1	3–4
Ni^{2+}	1.8	1.7	5
Zn^{2+}	2.2	—	4–5
UO_2^{2+}	3.2	—	—
Fe^{3+}	8.9	—	—
Al^{3+}	9.4	—	—
La^{3+}	10.5	—	50–60
Ce^{3+}	11.0	—	—

* On the basis of shifts of the Na^+ conductance *versus* voltage curves along the voltage axis[24].

The effects of various monovalent cations on Ca^{2+} binding to phospholipids were also examined. Reversal of the Na^+ to K^+ ratio in the aqueous solution or omission of all monovalent cations had a negligible effect on the binding of Ca^{2+} to phosphatidyl serine. Replacement of 116 mM NaCl in the aqueous solution by 116 mM of LiCl, RbCl or CsCl also had no effect.

3. Binding of cationic drugs to phospholipids and effect of cationic drugs on Ca^{2+} binding

In a previous communication[10], the binding of [^{14}C]procaine to phosphatidyl serine was described. Procaine also binds to phosphatidyl ethanolamine, as shown in Table II. Calculations of drug binding to phospholipids were based on the differences in distribution of drug between organic (chloroform) and aqueous phases in the absence (O/A) and presence (O'/A') of phospholipid. The increased amount of drug in the organic phase in the presence of phospholipid (O' — A'O/A) was taken as the

TABLE II

BINDING OF [^{14}C]PROCAINE TO PHOSPHATIDYL ETHANOLAMINE: EFFECT OF DIVALENT CATIONS*

*Ion***	*Distribution ratio****	*Procaine bound (μmole)*	*Inhibition (%)*
Control (no phospholipid)			
—	0.160	—	—
With 1 mg/ml phosphatidyl ethanolamine			
—	0.231	0.106	—
Mg^{2+}	0.199	0.057	46
Ca^{2+}	0.187	0.039	63
Ni^{2+}	0.161	0.000	100

* The initial concentration of procaine in the aqueous solution was 2.0 mM.

** Divalent cation was omitted from the original aqueous solution but was added in 1 mM concentration as shown.

*** The distribution ratio of [^{14}C]procaine was negligibly affected by the addition of divalent cations in the absence of lipid.

amount of drug bound to the phospholipid. Since no readily observable changes occurred in the relative volumes of the two phases as a result of adding salts or drugs to the system, it appears highly probable that the distribution ratios as measured could be directly compared.

FEINSTEIN's[12] observation that the binding of Ca^{2+} to phospholipids is inhibited by procaine and tetracaine has been confirmed in our laboratory[10], and similar results have been obtained with a number of other local anesthetics.

Several tropine and lidocaine analogues were tested because of the opportunity available to compare their effects on phospholipid ion binding with their

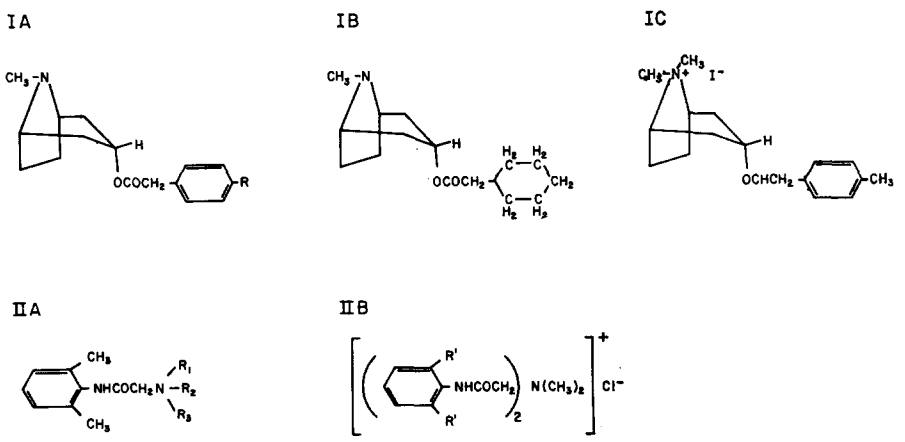

Fig. 5. Structural formulae of some tropine esters and lidocaine derivatives. I. Tropine esters. A. R=CH_3: Tropine *p*-tolyl acetate (TPTA); R=NO_2: Tropine *p*-nitrophenyl acetate (TPNφA); R=Cl: Tropine *p*-chlorophenyl acetate (TPClφA); R=H: Tropine phenyl acetate (TPA). B. Tropine cyclohexyl acetate (TPCHA). C. Tropine *p*-tolyl acetate methiodide (TPTA MeI). II. Lidocaine derivatives. A. R_1, R_2 = C_2H_5; R_3 = H: Lidocaine (Cl^-); R_1, R_2, R_3 = C_2H_5: QX-314 (Br^-). B. R′ = CH_3: QX-523 (Cl^-); R′ = H: QX-572 (Cl^-).

TABLE III

EFFECT OF TROPINE ESTERS AND LIDOCAINE ANALOGUES ON Ca^{2+} BINDING BY PHOSPHOLIPIDS

Abbreviations: see legend to Fig. 5.

Drug	*Concn. (mM)*	*Ca^{2+} bound to phosphatidyl serine (µmole)*	*Inhibition (%)*	*Ca^{2+} bound to phosphatidyl ethanolamine (µmole)*	*Inhibition (%)*	*Anesthetic effect**	
						Frog node[26,**]	*Lobster axon*[19,27]
Control	—	0.776	—	0.056	—	—	—
TPClφA	1.25	0.742	4	0.050	11	o	o
TPA	1.25	0.503	35	0.004	93	+	N.T.
TPNφA	1.25	0.492	36	0.004	93	+	N.T.
TPTA	1.25	0.471	39	0.002	96	+	+
TPTA MeI	1.25	0.427	45	0.000	100	+	+
TPCHA	1.25	0.537	31	0.004	93	+	N.T.
Control	—	0.848	—	0.058	—	—	—
Lidocaine	2.0	0.503	41	0.013	78	+	N.T.
	0.5	0.771	9	0.037	36		
QX-314 Br^-	2.0	0.630	26	0·052	10	o	N.T.
	0.5	0.804	5	0.056	3		
QX-523 Cl^-	2.0	0.233	72	0.006	90	+	N.T.
	0.5	0.745	12	0.014	76		
QX-572 Cl^-	2.0	0.227	73	0.007	88	+	N.T.
	0.5	0.709	13	0.013	78		

* o = no effect.
\+ = effective peripheral nerve block when applied locally, *in vitro*.
N.T. = not tested.
** C. CAMOUGIS, personal communication.

effects on peripheral nerve. The analogues which blocked peripheral nerve were also the most effective inhibitors of Ca^{2+} binding to phospholipids (Table III). These agents also had a greater binding affinity for phosphatidyl serine (Table IV).

TABLE IV

BINDING OF CATIONIC DRUGS TO PHOSPHATIDYL SERINE AT pH 7.4*

Abbreviations: see legend to Fig. 5.

Drug	*O/A***	*O'/A'***	*Drug "bound"**** *(µmole)*
TPTA	2.5	3.4	0.39
TPTA MeI	1.9	3.6	0.68
TPClφA	0.1	0.1	0.00
Lidocaine	0.4	0.9	0.49
QX-314 Br^-	0.3	0.6	0.35
QX-523 Cl^-	0.7	1.7	0.74

* Drug concentrations measured on the basis of ultraviolet absorption at 265 mµ, in the aqueous phase. Initial drug concentration in the aqueous phase was 2.0 mM. $CaCl_2$ concentration was 1.0 mM.
** Organic–aqueous phase drug concentration in the absence of (O/A) and in the presence of (O'/A') phosphatidyl serine.
*** µmole of drug bound to 2 mg of phosphatidyl serine.

TABLE V

EFFECT OF ANIONIC DRUGS ON Ca^{2+} BINDING BY PHOSPHOLIPIDS

Drug	*Concn. (mM)*	*pH*	*Phospholipid*	*Ca^{2+} "bound" (μmole)*	*Change (%)*
Pentobarbital	2.0	8.0	—	0.008	—
Pentobarbital	0.5	8.0	Phosphatidyl serine	0.887	+6
Pentobarbital	2.0	8.0	Phosphatidyl serine	0.911	+9
Pentobarbital	0.5	8.0	Phosphatidyl ethanolamine	0.069	+10
Pentobarbital	2.0	8.0	Phosphatidyl ethanolamine	0.143	+130
Diphenylhydantoin	2.0	8.0	—	0.003	—
Diphenylhydantoin	0.5	8.0	Phosphatidyl serine	0.894	+7
Diphenylhydantoin	2.0	8.0	Phosphatidyl serine	0.906	+9
Diphenylhydantoin	0.5	8.0	Phosphatidyl ethanolamine	0.070	+12
Diphenylhydantoin	2.0	8.0	Phosphatidyl ethanolamine	0.137	+123

4. Binding of anionic drugs to phospholipids and the effect of anionic drugs on Ca^{2+} binding to phospholipids. Effect of imidazole on Ca^{2+} binding

Certain anionic drugs such as the barbiturates may also bind to phospholipids *in vitro*[10]. In contrast to the cationic drugs just discussed, the anionic drugs enhance the binding of Ca^{2+} to phospholipids; conversely, the binding of ^{14}C-labeled anionic drug is increased by the addition of polyvalent cations, as shown in Tables V and VI (see also ref. 10 for data on thiopental, pentobarbital and the anionic detergent, sodium lauryl sulfate).

TABLE VI

EFFECT OF DIVALENT CATIONS ON BINDING OF ANIONIC DRUGS TO PHOSPHOLIPIDS

Drug	*O/A**	*O'/A'**	*Cation*	*Concn. (mM)*	*Phospholipid*	*Drug "bound" (μmole)*
Phenobarbital	1.7	2.4	—	—	Phosphatidyl serine	0.425
Phenobarbital	1.6	2.4	Ca^{2+}	1.0	Phosphatidyl serine	0.478
Phenobarbital	2.3	5.0	Ni^{2+}	1.0	Phosphatidyl serine	0.978
Phenobarbital	1.7	1.8	—	—	Phosphatidyl ethanolamine	0.082
Phenobarbital	1.6	1.9	Ca^{2+}	1.0	Phosphatidyl ethanolamine	0.153
Phenobarbital	2.3	3.5	Ni^{2+}	1.0	Phosphatidyl ethanolamine	0.575
Diphenylhydantoin	15.6	15.2	—	—	Phosphatidyl serine	0.000
Diphenylhydantoin	13.2	14.8	Ca^{2+}	1.0	Phosphatidyl serine	0.226
Diphenylhydantoin	13.4	16.0	Ni^{2+}	1.0	Phosphatidyl serine	0.323
Diphenylhydantoin	15.6	15.2	—	—	Phosphatidyl ethanolamine	0.000
Diphenylhydantoin	13.2	14.5	Ca^{2+}	1.0	Phosphatidyl ethanolamine	0.185
Diphenylhydantoin	13.4	15.0	Ni^{2+}	1.0	Phosphatidyl ethanolamine	0.213

* Distribution ratios in the absence of phospholipid (O/A), and in the presence of lipid (O'/A'), see text.

Recently, SANDOW AND ISAACSON[28] found that imidazole mimics the action of certain polyvalent cations on skeletal muscle by increasing the twitch-tetanus ratio. Imidazole, like the barbiturates, increases the binding of Ca^{2+} to phosphatidyl serine (Table VII).

TABLE VII

EFFECT OF IMIDAZOLE ON Ca^{2+} BINDING TO PHOSPHATIDYL SERINE

Imidazole (mM)	*Ni^{2+} (mM)*	*Ca^{2+} bound (μmole)*	*Change (%)*
Controls (no phospholipid)			
0.0	0.0	0.001	—
2.0	0.0	0.014	—
2.0	0.5	0.010	—
With 1 mg/ml phosphatidyl-L-serine			
0.0	0.0	0.735	—
0.0	0.5	0.642	−13
2.0	0.0	0.811	+9
2.0	0.5	0.787	+6

5. Effect of non-ionic drugs on Ca^{2+} binding to phospholipids

Several higher alcohols (C_4–C_8) and two samples of octane were tested for their effects on Ca^{2+} binding to phospholipids. Millimolar concentrations of these agents had a negligible effect. At molar concentrations they reduced slightly the binding of Ca^{2+} to phosphatidyl serine. However, this reduction of Ca^{2+} binding may not be primarily a direct result of electrostatic interaction between the phospholipid polar groups and the non-ionic drugs but rather a consequence of changes in the properties of the lipid solvent.

6. Effect of SH-reagents and SH-group inhibitors on divalent ion binding to phospholipids

Studies on the effects of SH-reagents and SH-group inhibitors on excitable tissues have led to the suggestion[29–33] that SH-groups may be directly involved in

TABLE VIII

EFFECT OF SH-REAGENTS AND SH-INHIBITORS ON BINDING TO PHOSPHOLIPIDS AT pH 7.4

Reagent	*Concn. (mM)*	*Phospholipid*	*Ca^{2+} bound (μmole)*	*Change (%)*
Control	—	—	0.001	—
Control	—	Phosphatidyl serine	0.757	—
Control	—	Phosphatidyl ethanolamine	0.062	—
Ni^{2+}	0.5	Phosphatidyl serine	0.546	−28
Cysteine-HCl	2.0	—	0.001	0
Cysteine-HCl	2.0	Phosphatidyl serine	0.731	−3
Cysteine-HCl + Ni^{2+}	2.0 + 0.5	Phosphatidyl serine	0.761	+1
PHMB	2.0	—	0.012	—
PHMB	2.0	Phosphatidyl serine	0.905	+20
PHMB	0.5	Phosphatidyl serine	0.791	+4
PHMB	2.0	Phosphatidyl ethanolamine	0.148	+120
N-Ethyl maleimide	2.0	—	0.000	—
N-Ethyl maleimide	2.0	Phosphatidyl serine	0.752	−1
N-Ethyl maleimide	0.5	Phosphatidyl serine	0.747	−1
N-Ethyl maleimide	2.0	Phosphatidyl ethanolamine	0.061	0

the molecular interactions underlying excitation. For example, cysteine–HCl, which by itself has a negligible effect on nerve activity, will antagonize "plateau formation" induced by various divalent ions such as Ni^{2+} and Co^{2+} in the frog nerve action potential[31,33]. Cysteine–HCl (Table VIII) has relatively little effect on Ca^{2+} binding to phosphatidyl serine, while Ni^{2+} (Table I) antagonizes the binding of Ca^{2+} to phospholipid. When cysteine–HCl and Ni^{2+} are both present in the aqueous phase, no decrease in Ca^{2+} binding occurs (Table VIII).

Also shown in Table VIII are data for two SH-group inhibitors, PHMB and *N*-ethyl maleimide. *N*-Ethyl maleimide, which has no effect on peripheral nerve[29], does not affect Ca^{2+} binding to phospholipids. On the other hand, PHMB, which may act as a blocking agent in peripheral nerve[29,34], appears to increase the binding of Ca^{2+} to phosphatidyl serine, and thus acts somewhat like the barbiturates.

DISCUSSION

1. Comparison of the biphasic distribution method with the lipid monolayer method for determining the binding properties of lipids

As already pointed out, the curve of pH *versus* Ca^{2+} binding to phosphatidyl serine is similarly shaped, whether the monolayer technique[15] or the biphasic distribution technique (*i.e.*, the method used here) is employed.

A second comparison between these two techniques comes from data on the competition between divalent cations and cationic anesthetics for binding to phospholipids. Data from the biphasic distribution studies have been described at length (refs. 10 and 12 and the results above). SKOU's[17] data provide evidence for a similar type of competition when the monolayer technique is employed. His Fig. 1 shows that cocaine was significantly more effective in increasing monolayer surface pressure when NaCl, KCl, and $CaCl_2$ were omitted from the hypophase. The fact that divalent cations are bound roughly a thousand-fold more strongly to phospholipids than are monovalent cations[35] suggests that omission of Ca^{2+} is likely the most significant factor in SKOU's experiments.

Nevertheless, certain problems in interpreting data from the biphasic distribution studies must be considered. It seems likely that the phospholipid molecules are arranged in micelles within the chloroform phase. Possibly, some drugs may disrupt or otherwise affect the micellar arrangement and thereby affect the ion-binding properties of the lipid. The effects of adding alcohols or in other ways varying the organic solvents are thus particularly difficult to evaluate. Errors due to the possible movement of lipid into the aqueous phase (thereby giving abnormally low binding values) must also be considered, although, as mentioned in METHODS, this did not appear to be a significant problem when 2:1 chloroform–methanol was employed.

2. Competition between cationic drugs and divalent cations. Comparison with studies on peripheral nerve

Correlation between relative peripheral nerve-blocking activity and antagonism of Ca^{2+} binding to phospholipid has now been demonstrated for a number of local anesthetics (refs. 10 and 12 and RESULTS above). The data on the tropine esters and lidocaine anologues (Table III) provide another example of the parallelism between the phospholipid and the peripheral nerve studies. The two compounds

which were ineffective as nerve-blocking agents, tropine *p*-chlorophenyl acetate and the *N*-ethyl quaternary analogue of lidocaine, were also the least effective inhibitors of Ca^{2+} binding. These two compounds also have relatively low organic-to-aqueous phase distribution ratios and are only relatively weakly bound to phosphatidyl serine. Such observations are in agreement with the well known correlation between anesthetic potency and molecular polarizability or solubility in media with low dielectric constants[17,36–39]. They suggest that the classical notions of MEYER[40,41] and OVERTON[42] on the role of lipid solubility must still be taken into consideration if we are to gain insight into the molecular mechanisms underlying anesthesia.

In addition, the polar groups of ionizable anesthetics play a fundamental role, as demonstrated by studies on the effect of pH on local anesthetic activity[43] and the comparison of lidocaine analogues[38]. Further evidence for the involvement of polar groups comes from the observed antagonism between procaine-like anesthetics and divalent cations such as Ca^{2+}, Co^{2+} and Ni^{2+} (refs. 18 and 19 review this subject). This antagonism seems comparable to the effects observed *in vitro* in these phospholipid studies. Also noteworthy is the correlation between the relative binding affinity of polyvalent cations, and their effectiveness in maintaining excitability in peripheral nerve when substituted for Ca^{2+} (Table I).

3. Drugs which increase the binding of Ca^{2+} to phospholipids; correlation with studies on excitable tissues

Phenobarbital, thiopental, pentobarbital, diphenylhydantoin, imidazole, sodium lauryl sulfate, and PHMB, all of which mimic some of the actions of divalent cations on excitable tissues[28,32,44–47], also increase the binding of Ca^{2+} to phospholipids (Tables V and VIII and *cf.* ref. 10)—another similarity between the phospholipid experiments and the pharmacology of excitable membranes.

4. SH-groups and the excitation process: inadequacy of available data

The data on Ca^{2+}, Ni^{2+} and cysteine in Table VIII provide evidence that the SH-reagents may merely be acting as chelators of certain metal ions (Ni^{2+} in this case). The observed action of PHMB in increasing Ca^{2+} binding as well as in antagonizing the effect of Ni^{2+}, while *N*-ethyl maleimide is entirely without effect in the phospholipid system, suggests that the supposed specificity of PHMB and *N*-ethyl maleimide as SH-group inhibitors may have been overemphasized. These data thus appear to contradict the reasoning used to imply a direct role for SH-groups in excitation processes[29–33], and support the suggestion[28] that phosphate groups are better candidates than SH-groups for the ion exchange sites associated with the excitation of biological membranes.

In this discussion, no distinction has been drawn between the phosphate groups of phosphatidyl ethanolamine and the combination of phosphate and carboxyl groups of phosphatidyl serine. However, as noted above, the relative differences in binding affinity of various polyvalent cations to phosphatidyl ethanolamine are much less marked than to phosphatidyl serine. It may be therefore that the polar heads of phosphatidyl serine are more likely to be involved in ion exchange processes in biological membranes. Other possible sources of phosphate groups whose ion exchange characteristics we have not explored include phosphatidyl choline, and

phosphatidyl and polyphosphatidyl inositide, although phosphatidyl choline is known not to bind calcium[15].

While these data suggest that phosphate (and possibly carboxyl) groups have many of the characteristics to be expected of some biological membrane ion exchangers, the evidence is only circumstantial; we are, nevertheless, unaware of any other good candidates for this role.

5. Ion and drug interactions with phospholipids; possible role in anesthesia at the molecular level

Correlations between phospholipid studies and properties of peripheral nerve provide support for a model concerned with the cellular action of anesthetics[10]. According to this model, the nerve membrane is visualized as a lipid bilayer, coated by protein on both its inner and outer surfaces (*cf.* the Davson–Danielli model), with ion exchanger phospholipids making up a significant proportion of at least the externally facing lipid layer[48]. This arrangement of the outer layer is therefore analogous to a monolayer of mixed lipids and phospholipids. Possible reactions which the polar heads of these phospholipids could undergo are depicted diagrammatically in Fig. 6. No intimations regarding stoichiometry are implied; there are no good data favoring either a single polar group, or a polar group cluster, as "the ion exchange site" in the biological membrane. As shown in A and D of Fig. 6, we might expect that "at rest", most polar groups would be tied up by divalent cations. The proportion of polar groups bound in this manner would be a function of the divalent cation concentration and the binding constant for the particular cation involved. During depolarization, the change in the electric field across the polar heads might be ex-

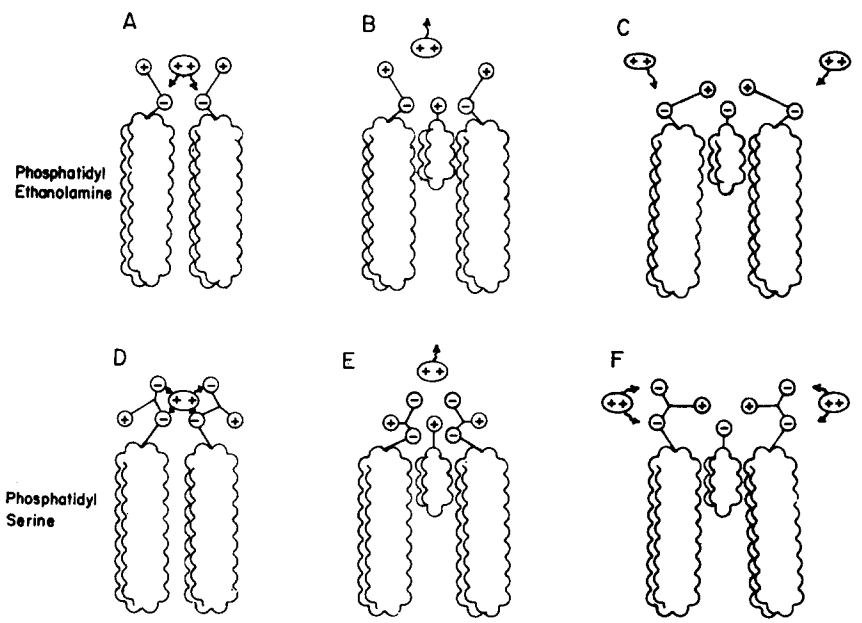

Fig. 6. Molecular models demonstrating interactions of phospholipids, divalent cations and anionic and cationic drugs (see text).

pected to change the orientation of unbound polar heads, thereby changing their ion exchange properties. Polar heads could then be available for ion exchange reactions involving Na^+ and/or K^+, thereby accounting for the Na^+ and K^+ currents associated with the nerve action potential[16].

We would expect cationic drugs such as procaine to compete with the divalent ions for binding to the phospholipid polar heads. The lipid-soluble properties of these drug molecules would help them to compete successfully, since the lipid-soluble moiety would likely be inserted between the fatty acid chains of the phospholipids, as in Fig. 6B and E.

The lipid-soluble characteristics of anionic drugs would also favor their insertion between the phospholipid fatty acid chains (C and F of Fig. 6). In this case, however, the increase in net negative charge in the region of the phospholipid polar groups, would be expected to result in increased divalent cation binding. Changing the electric field across the polar heads, by depolarization, should result in the release of divalent cations and some of the anionic or cationic drug molecules. The lipid-soluble nature of the drug molecules would, however, keep most of these molecules within the lipid layer. Non-polar molecules such as the alcohols, diethyl ether and fluothane might also be expected to dissolve in the lipid portion of the membrane. The non-polar drugs would have little direct effect on the polar heads, and would be relatively unaffected by changes in the electric field.

The electrophysiological correlates which might be anticipated as a result of the squeezing of drug molecules in between the lipid molecules of the membrane include an increase in the electrical resistance across the membrane, with reduction of membrane Na^+ and K^+ conductances. Reduction of maximum Na^+ and K^+ conductances has indeed been found to occur in nerve as a consequence of treatment with cationic[18,49,50], anionic[44], and non-ionic drugs[51,52]. Agents which directly affect the polar groups, *i.e.*, the polyvalent cations and ionic drugs, would be expected to affect the time parameters for the conductance changes and the position of the membrane potential-*versus*-voltage curves on the potential axis. Again, electrophysiological data are consistent; polyvalent cations[53], cationic drugs[19] and anionic drugs[44,45] all affect these parameters. Thus, the anionic and cationic anesthetics have effects which likely involve both polar group interactions and the presence of a lipid-soluble moiety in the membrane lipid layer. The alcohols do not affect significantly the rate parameters for the turning on of the Na^+ conductance[52] as expected for lipid-soluble drugs having no polar groups to interact with the phospholipid polar heads.

Imidazole does not appear to fit into the above structural categories. This nucleophilic molecule would be expected to neutralize the net positive charge on the amino group of the phosphatidyl serine (for example), thereby, in effect, increasing the net negative charge in the region of the polar heads so that increased Ca^{2+} binding occurs. Since the imidazole molecule is small and lipid-soluble, one might expect it to mimic the effects of polyvalent cations[28] but not necessarily to have the resistance-increasing effects of drugs with large lipid-soluble moieties.

Thus phospholipids, with the ion exchange properties discussed above, may provide a reasonable model for interpretation of polyvalent cation and anesthetic drug action on the nerve membrane.

ACKNOWLEDGEMENTS

Mr. J. A. SCHEMINSKE provided excellent technical assistance with these experiments. The tropine esters were very kindly supplied by Dr. S. FRIESS, of the Naval Medical Research Institute, and Dr. G. CAMOUGIS, of Astra Pharmaceutical Products, Inc., generously supplied the lidocaine analogues. Very much appreciated were the gifts of drugs from Abbott Laboratories and Parke, Davis and Company. The author is particularly indebted to Dr. D. E. GOLDMAN for advice and encouragement throughout the course of these studies and to Dr. S. L. FRIESS for many helpful suggestions and criticisms during the course of these studies, and for suggestions on the manuscript. From the Bureau of Medicine and Surgery, Navy Department Research Task MR-005.08-0020.02. The opinions or assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

REFERENCES

1 E. OVERTON, *Vierteljahresschr. Naturforsch. Ges. Zürich*, 40 (1895) 159.
2 E. OVERTON, *Jahrb. Wiss. Botan.*, 34 (1900) 669.
3 E. GORTER AND F. GRENDEL, *J. Exptl. Med.*, 41 (1925) 439.
4 H. DAVSON AND J. F. DANIELLI, *The Permeability of Natural Membranes*, Cambridge University Press, Cambridge, 1943, p. 60.
5 J. B. FINEAN, R. COLEMAN AND W. A. GREEN, *Ann. N.Y. Acad. Sci.*, 137 (1966) 414.
6 J. FOLCH-PI AND F. N. LE BARON, in D. RICHTER, *Metabolism of the Nervous System*, Pergamon Press, New York, 1957, p. 67.
7 J. D. ROBERTSON, *Ann. N.Y. Acad. Sci.*, 137 (1966) 421.
8 E. D. KORN, *Science*, 153 (1966) 1491.
9 B. I. ROOTS AND P. V. JOHNSTON, *Biochem. J.*, 94 (1965) 61.
10 M. P. BLAUSTEIN AND D. E. GOLDMAN, *Science*, 153 (1966) 429.
11 V. BREYER AND G. QUADBECK, *J. Neurochem.*, 13 (1966) 493.
12 M. B. FEINSTEIN, *J. Gen. Physiol.*, 48 (1964) 357.
13 H. KIMISUKA AND K. KOKETSU, *Nature*, 196 (1962) 995.
14 H. A. NASH AND J. M. TOBIAS, *Proc. Natl. Acad. Sci. U.S.*, 51 (1964) 476.
15 E. ROJAS AND J. M. TOBIAS, *Biochim. Biophys. Acta*, 94 (1965) 394.
16 D. E. GOLDMAN, *Biophys. J.*, 4 (1964) 167.
17 J. C. SKOU, *Acta Pharmacol. Toxicol.*, 10 (1954) 325.
18 M. P. BLAUSTEIN AND D. E. GOLDMAN, *J. Gen. Physiol.*, 49 (1966) 1043.
19 D. E. GOLDMAN AND M. P. BLAUSTEIN, *Ann. N.Y. Acad. Sci.*, 137 (1966) 967.
20 M. P. BLAUSTEIN AND D. E. GOLDMAN, *Federation Proc.*, 25 (1966) 511.
21 G. V. MARINETTI AND E. STOTZ, *Biochim. Biophys. Acta*, 21 (1956) 168.
22 J. E. GARVIN AND L. M. KARNOVSKY, *J. Biol. Chem.*, 221 (1956) 211.
23 J. E. GARVIN AND L. M. KARNOVSKY, in G. POPJAK AND E. LE BRETON, *Biochemical Problems of Lipids*, Butterworths, London, 1956, p. 14.
24 M. P. BLAUSTEIN AND D. E. GOLDMAN, *Abstr. 10th Ann. Biophys. Soc. Meeting*, 1966, p. 132.
25 F. HELFFERICH, *Ion Exchange*, McGraw Hill, New York, 1962.
26 S. L. FRIESS, B. R. WHITCOMB, C. D. THRON, R. C. DURANT, L. J. REBER AND R. N. PATTERSON, *Arch. Biochem. Biophys.*, 95 (1961) 85.
27 M. P. BLAUSTEIN, *J. Gen. Physiol.*, in the press.
28 A. SANDOW AND A. ISAACSON, *J. Gen. Physiol.*, 49 (1965) 937.
29 H. M. SMITH, *J. Cellular Comp. Physiol.*, 51 (1958) 161.
30 H. TAKAHASHI, T. MURAI AND T. SASAKI, *Nature*, 182 (1958) 1675.
31 H. TAKAHASHI, T. MURAI AND T. SASAKI, *Japan. J. Physiol.*, 10 (1960) 280.
32 H. TAKAHASHI, K. WATANABE, N. MORITA AND T. TOMIKAWA, *Japan. J. Physiol.*, 6 (1956) 225.
33 K. UCHIZONO AND Y. MATSUMOTO, *Japan. J. Physiol.*, 8 (1958) 319.
34 T. NARAHASHI, *J. Cellular Comp. Physiol.*, 64 (1964) 73.
35 E. J. ROJAS, J. Y. LETTVIN AND W. F. PICKARD, *Nature*, 209 (1966) 886.
36 D. AGIN, L. HERSH AND D. HOLTZMAN, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 952.
37 J. A. CLEMENTS AND K. M. WILSON, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 1008.

38 N. LÖFGREN, *Studies on Local Anesthetics: Xylocaine, a New Synthetic Drug*, Ivar Haeggströms, Stockholm, 1948.
39 J. C. SKOU, *Biochim. Biophys. Acta*, 30 (1958) 625.
40 H. MEYER, *Arch. Exptl. Pathol. Pharmakol.*, 42 (1899) 109.
41 H. MEYER, *Arch. Exptl. Pathol. Pharmakol.*, 46 (1901) 338.
42 E. OVERTON, *Studien über die Narkose zugleich ein Beitrag zur algemeinen Pharmakologie*, G. Fischer, Jena, 1901.
43 J. M. RITCHIE AND P. GREENGARD, *Ann. Rev. Pharmacol.*, 6 (1966) 405.
44 M. P. BLAUSTEIN, *Abstr. 2nd Intern. Congr. Pure Applied Biophys.*, 1966, 411.
45 U. KISHIMOTO AND W. J. ADELMAN, JR., *J. Gen. Physiol.*, 47 (1964) 975.
46 S. KOREY, *Proc. Soc. Expt. Biol. Med.*, 76 (1951) 297.
47 J. E. P. TOMAN, *Electroenceph. Clin. Neurophysiol.*, 1 (1949) 33.
48 C. F. DOGGENWEILER AND S. FRENK, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 425.
49 A. M. SHANES, W. H. FREYGANG, H. GRUNDFEST AND E. AMATNIEK, *J. Gen. Physiol.*, 42 (1959) 793.
50 R. E. TAYLOR, *Am. J. Physiol.*, 196 (1959) 1071.
51 C. M. ARMSTRONG AND L. BINSTOCK, *J. Gen. Physiol.*, 48 (1964) 265.
52 J. W. MOORE, W. ULBRICHT AND M. TAKATA, *J. Gen. Physiol.*, 48 (1964) 279.
53 B. FRANKENHAEUSER AND A. L. HODGKIN, *J. Physiol. London*, 138 (1957) 218.