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LANGMUIR ADSORPTION BEHAVIOR WITH CATION-INDUCED VOLTAGES ACROSS BIMOLECULAR LIPID MEMBRANES*

THOMAS H. MASSEY** AND ROBERT J. McCOLLOCH
Department of Biochemistry, University of Wyoming, Laramie, Wyo. 82070 (U.S.A.)
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SUMMARY

Potentials were induced across bimolecular lipid membranes with asymmetric concentrations of alkali, alkaline earth, Group IIb and some other transition metal chlorides. The transmembrane potential increments decreased with linear increments of cation concentration indicating a Langmuir adsorption behavior. Using the assumptions employed in the Langmuir adsorption equation a voltage adsorption equation was derived for cation adsorption to a membrane site with or without a subsequent transport step through the membrane.

Double reciprocal plots of voltage (ordinate) and cation concentration (abscissa) resulted in straight lines with definite voltage maxima obtained at extrapolation to infinite cation concentrations. The abscissa intercepts were interpreted as reciprocal dissociation constants.

The monovalent cations showed various voltage maxima in the order of $Na^+ = K^+ > Cs^+ > Li^+$, but displayed the same dissociation constant ($4 \cdot 10^{-3}$ M). Most of the divalent cations showed similar voltage maxima (about 45 mV) and dissociation constants ($1 \cdot 10^{-4}$ M).

The voltage maxima and dissociation constants for Ca^{2+} showed no variation over a pH range of 5.0 to 8.5. At pH's lower than 5.0 the dissociation increased and the voltage decreased. No voltage was observed below pH 3.5.

Equal concentrations of the alkali chlorides on both sides of the membrane repressed the Ca^{2+}-induced voltage. The effect was described with a Langmuir-type voltage adsorption equation in which competitive binding of the alkali cations with Ca^{2+} occurred.

INTRODUCTION

In recent years a new area of research has developed involving the formation and study of phospholipid membranes in aqueous solutions[1-4]. Because of the marked similarity of the bimolecular lipid membrane to membranes occurring in living cells,

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** Present address: Department of Biochemistry, Michigan State University, East Lansing, Mich. 48823, U.S.A.

they have been used as models to study the functions of natural membranes. One of the more important properties of plasmic membranes is the production of transmembrane voltages, the inside of the cell being negative with respect to the outside. Consequently there has been a large interest in producing trans-bimolecular lipid membrane potentials. Generally the unmodified bimolecular lipid membrane has been incapable of producing potentials by means of concentration gradients of various salts[4]. However, the addition of certain substances to either the aqueous phase or lipid mixture used for making the membranes has given the membrane a selective permeability to some cations and H^+ allowing the development of appreciable potentials.

MUELLER AND RUDIN[5] and LEV AND BUZHINSKY[6] independently reported the effect of valinomycin and other macrocyclic antibiotics on the permeability of K^+ in which large decreases in resistance occurred. Potentials appeared with concentration gradients of K^+ or if K^+ was pitted against Na^+ (ref. 5). The side containing the highest concentration of K^+ was negative.

Another group of substances demonstrating large drops in bimolecular lipid membrane resistance and used to develop transmembrane potentials include compounds capable of uncoupling oxidative phosphorylation in mitochondria, 2,4-dinitrophenol, carbonyl cyanide *m*-trifluorophenylhydrazone, carbonyl cyanide *m*-chlorophenylhydrazone, and dicoumarol. HOPFER *et al.*[7] showed that in the presence of a pH gradient the optimum pH of the buffers was approximately the same as the p*K* of ionization of the uncouplers. The side of the membrane having the lowest pH was negative. In this case the carrier was presumed to be protons.

I^- has been found by LÄUGER *et al.*[8], to be able to induce not only large decreases in resistance but also high voltages across bimolecular lipid membrane. Of special interest was the ability of KNO_3, KCl, and NaCl to induce "inconstant and non-reproducible potentials". The sign of the voltage with regard to concentration gradient was not specified. The voltages were found to be proportional to the log of the ratio of the activities of I^- on the two sides of the membrane. More recently the voltage with Na^+ and K^+ has been studied extensively by HOPFER *et al.*[9]. The work also describes the variation of transmembrane potential with the log of the ratio of the activities of the cation on both sides of the membrane. The sensitivity of various negative and neutral charged phospholipids to cations was varied while a positively charged phospholipid showed a marked sensitivity only to Cl^- concentrations.

This paper attempts to present an alternate view to the description of cation-induced trans-bimolecular lipid membrane potentials by introducing the concept of Langmuir adsorption behavior. The result is that the degree of binding of a cation for a membrane site can be determined and expressed in the form of equilibrium constants.

MATERIALS AND METHODS

(*1*) *Preparation of the phospholipids*

Lecithin was isolated by a modification of the method of PANGBORN[10] in which the $CdCl_2$ precipitate, from an alcoholic extract of acetone-washed egg yolks (700 ml), was dissolved in chloroform and washed with water–ethanol (70:30, by vol.). The lecithin–chloroform solution, devoid of Cl^-, was dried and washed with acetone. The

acetone washes removed residual yellow from the precipitated lecithin yielding about 12 g of lecithin.

Phosphatidylethanolamine was also obtained from an alcoholic extract by the addition of 50 ml of 50 % $CaCl_2$ with subsequent overnight storage at 4°. The precipitate, which was very gummy and stuck to the sides of the flask, was dissolved in chloroform and washed the same as the lecithin preparation. After evaporating the chloroform the precipitate was washed with acetone removing residual yellow and yielding about 8 g of phosphatidylethanolamine.

Both the lecithin and phosphatidylethanolamine preparations were stored as solutions in absolute ethanol (100 mg/ml) under nitrogen and at —20°. Thin-layer chromatography by the method of ROUGHAN AND BATT[11] in a developing solvent of chloroform–methanol–acetic acid–water (85:15:10:3, by vol.) showed a single spot for the $CdCl_2$ precipitate with an R_F value of about 0.53 indicating reasonably pure lecithin and a major spot for the $CaCl_2$ precipitate with an R_F value of about 0.75 *plus* three small spots. The major spot was taken to be phosphatidylethanolamine and the impurities were possibly lecithin, lysolecithin, and phosphotidylinositol. The yields correspond to the two main phospholipid components of egg yolk[12].

The lipid mixture, from which the bimolecular lipid membranes were formed, was composed of 50 % lecithin and 50 % phosphatidylethanolamine in *n*-decane (40 mg/ml). All ethanol and chloroform were evaporated and *n*-decane was added to dissolve the phospholipid to make the final concentration. No ethanol was added to prevent gelling as this was not a major problem. The lipid preparation was routinely prepared 2 or 3 times daily.

Unless otherwise specified the buffers were composed of 5 mM Tris–HCl (pH 7.0). Tris was employed since it has been shown to have a low permeability to biological membranes[7,13]. The buffers were all gassed with nitrogen for 0.5 h to insure the complete removal of oxygen since HUANG *et al.*[14] had demonstrated that the presence of oxygen dramatically shortens the life of the bimolecular lipid membranes formed from phospholipids.

(2) *Instrumentation*

The experimental apparatus involved a simple series circuit with a Keithley electrometer Model 610 C (input resistance $10^{14}\,\Omega$), Ag–AgCl electrodes in 1 M KCl, and the membrane. The outer chamber was at ground. The membranes were formed on a 1-mm hole in a 5-ml Teflon cup supported in a square glass cell (80 ml) by a plexiglass lid. A small stirring rod fashioned from a piece of paper clip (1 cm), covered with polyethylene tubing fused at both ends was placed in the glass cell. The cell and electrodes, clamped over a magnetic stirrer, were all electrostatically shielded by a metal box with copper screen windows. The membranes were observed with a 10 × microscope and light projector. Voltage traces were recorded with a Heath recorder connected through a 5-kΩ helipot to the 1-mA, 3-V output of the electrometer.

(3) *Formation of the bimolecular lipid membrane*

The membranes were formed by injecting the lipid mix into the hole with a syringe and bent steel needle (20 gauge). Using the tip of the needle, part of the lipid was pulled away forming a colored film surrounded by a torus of bulk phase lipid.

The colored bands became arranged in a parallel fashion with the thinnest part occurring at the bottom and colored a creamy white. It was here that one or more black spots appeared increasing in size by pushing the colored film lipid aside as circular globs, bordering the black film. Eventually the black film completely displaced the colored film and in turn became surrounded by the torus of bulk phase lipid. If black spot formation did not occur, a voltage applied across the colored film would initiate the process. After each experiment the Teflon cup was washed with alcohol. There was no pretreatment of the hole with lipid before starting a new membrane.

(4) Additions to the outer chamber buffer

Solutions that were to be added to the outer buffer were injected by means of a syringe with a stainless steel needle and attached 3-inch piece of spaghetti tubing. A piece of aluminum was fitted to the syringe to act as a plunger stop, insuring accurate and reproducible delivery of 0.16 ml such that the solution injected was diluted by a factor of 0.16 ml/80 ml $= 2 \cdot 10^{-3}$. After injection the syringe was filled with buffer from the outer chamber to insure that there was no net volume change and the membrane was not stretched. The magnetic stirrer was turned on before injection.

(5) Mathematical model

In the data to be presented in this paper, the introduction of metal chloride salts into the aqueous phase on one side of the membrane resulted in the appearance of transmembrane potentials. The integrity of the membrane and stability of the potential was found to be quite adequate over the time span of all of the experiments (10–15 min). It was observed that with each incremental increase of cation concentration the voltage increased but with decreasing increments. With this phenomenon indicating a possible LANGMUIR[15] adsorption behavior it was decided to plot the reciprocal of the voltage for a particular cation concentration *versus* the reciprocal of that concentration. As is seen the plots result in straight lines. The following is an attempt to rationalize the results assuming an adsorption process for the cation with or without a subsequent transport step across the membrane.

For a cation C being adsorbed to a site on a membrane M in aqueous solution

$$C + M \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CM$$

one can write an adsorption equation

$$y = \frac{y_m \cdot [C]}{[C] + K}$$

where y is the number of cations adsorbed and y_m is the maximum number that can be adsorbed and where [C] = cation concentration; $K = k_2/k_1$.

If one assumes that the voltage induced across the membrane is proportional to only the number of cations bound to the membrane, that is if

$y \propto v$ = voltage

and

$y_m \propto V$ = maximum voltage

then

$$v = \frac{V \cdot [C]}{K + [C]} \tag{1}$$

For a cation that is adsorbed to a site on one side of the membrane, transported across and released on the other side as P

$$C + M \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CM \overset{k_3}{\rightarrow} M + P$$

and if a steady state is assumed, that is

$$\frac{d[CM]}{dt} = 0$$

and

$$K = \frac{k_2 + k_3}{k_1}$$

then

$$r = \frac{R \cdot [C]}{K + [C]}$$

where $r = -d[C]/dt$ and $R =$ maximum r.

It is noted that the mathematics used here are similar to the derivations for the kinetics of enzyme catalyzed reactions[16].

Since the rate of transport of cation across the membrane is the same as current and current is proportional to voltage at constant resistance, that is

$$r \propto v$$

$$R \propto V$$

then

$$v = \frac{V \cdot [C]}{K + [C]} \tag{2}$$

Eqns. 1 and 2 are seen to be identical and will be referred to as the voltage–adsorption equation. Inverting Eqn. 2

$$\frac{1}{v} = \frac{K}{V} \cdot \frac{1}{[C]} + \frac{1}{V}$$

It is seen that a double reciprocal plot of $1/v$ *versus* $1/[C]$ should be linear.

For purposes of extrapolation, when

$$1/[C] = 0$$

then

$$v = V$$

and when

$$1/v = 0$$

then

$$-[C] = K$$

If a second cation is present on both sides of the membrane

$$C + M \underset{k_2}{\overset{k_1}{\rightleftharpoons}} CM \overset{k_3}{\rightarrow} M + P$$

$$A + M \underset{k_5}{\overset{k_4}{\rightleftharpoons}} AM$$

and assuming a quasi-equilibrium state, the following modification of the adsorption equation can be derived

$$\frac{1}{v} = \frac{K_c(1 + [A]/K_a)}{V} \cdot \frac{1}{[C]} + \frac{1}{V}$$

where

$$K_a = \frac{k_5}{k_4}, \qquad K_c = \frac{k_2 + k_3}{k_1}$$

Again a double reciprocal plot of $1/v$ *versus* $1/[C]$ should give a straight line. Setting

$$\frac{1}{v} = 0$$

$$0 = 1 + [C]/K_c + [A]/K_a$$

or

$$-[C] = \frac{K_c}{K_a} \cdot [A] + K_c \tag{3}$$

Here a plot of the apparent negative concentration *versus* [A] should give a straight line with an intercept of K_c and a slope of K_c/K_a allowing a solution of K_a.

RESULTS

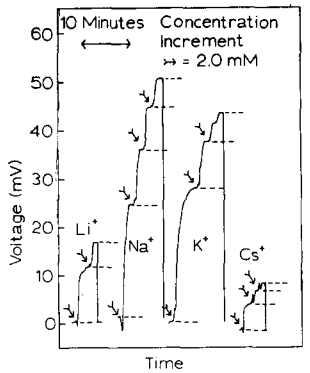
(1) *Monovalent and divalent cation chloride induced voltages*

The injection of solutions of the alkali metal ion chlorides into the outer chamber of the cell produced the voltage traces seen in Fig. 1. Both Na^+ and K^+ are equally able to induce large voltages while Li^+ and Cs^+ are much less effective. It is noted that the voltage was positive in the inner chamber or negative on the side of the membrane containing the metal cation. When the electrostatic shield was given a sharp blow the membrane was broken with simultaneous return of the voltage to zero indicating that only the membrane was responsible for the effect. The decreasing magnitude of the voltage increment with constant increments of cation concentration is apparent.

Plots of the reciprocal voltage *versus* the reciprocal cation concentration are shown in Fig. 2. It is immediately obvious that the points lie reasonably well on a straight line. The ordinate and abscissa intercepts are taken as the reciprocal voltage maxima and reciprocal dissociation constants, respectively. Interestingly, the abscissa intercepts for all four monovalent cations tested show roughly the same value, indicating similar dissociation constants as seen in Table I.

The voltage traces for the alkaline earth and Group IIb cations, in the presence of 5 mM NaCl on both sides of the membrane, are shown in Fig. 3 and all other divalent cations tested are shown in Fig. 4. The double reciprocal plots for the divalent cations shown in Figs. 5 and 6 give straight lines with most of the metals displaying similar ordinate intercepts indicating similar voltage maxima. The abscissa intercepts show some variation indicating differences in binding affinity to the membrane. The voltage maxima and dissociation constants for the various divalent cations tested, in the presence of 5 mM NaCl, are shown in Table I.

It is noted in Fig. 3 that divalent mercury did not display any voltage when



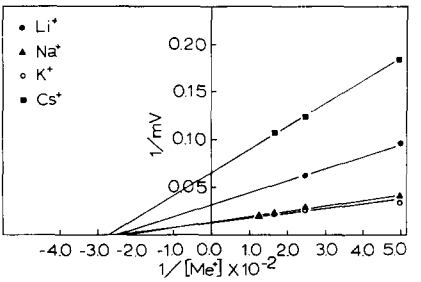


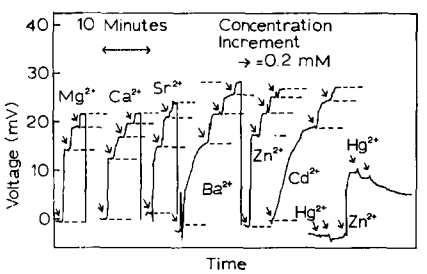
Fig. 1. Voltage traces for the alkali chlorides. Buffer was 5 mM Tris (pH 7.0).

Fig. 2. Reciprocal voltage *versus* reciprocal alkali ion concentration.

TABLE I

DISSOCIATION CONSTANTS AND VOLTAGE MAXIMA FOR VARIOUS CATIONS

Cation	*Buffer 5 mM Tris–HCl*		*Buffer 5 mM Tris–HCl, 5 mM NaCl*		*Corrected for no NaCl present (see* RESULTS, *Section 3)*	
	K (*M*)	*V* (*mV*)	*K* (*M*)	*V* (*mV*)	*K* × 0.41 (*M*)	*V* × 1.45 (*mV*)
Li^+	$4.07 \cdot 10^{-3}$	32				
Na^+	$4.26 \cdot 10^{-3}$	71				
K^+	$3.94 \cdot 10^{-3}$	71				
Cs^+	$3.93 \cdot 10^{-3}$	15				
Mg^{2+}			$1.85 \cdot 10^{-4}$	29	$0.76 \cdot 10^{-4}$	42
Ca^{2+}			$2.9 \cdot 10^{-4}$	33	$1.2 \cdot 10^{-4}$	48
Sr^{2+}			$2.9 \cdot 10^{-4}$	34	$1.2 \cdot 10^{-4}$	49
Ba^{2+}			$2.9 \cdot 10^{-4}$	42	$1.2 \cdot 10^{-4}$	67
Co^{2+}			$4.08 \cdot 10^{-4}$	33	$1.67 \cdot 10^{-4}$	48
Cu^{2+}			$2.67 \cdot 10^{-4}$	35	$1.1 \cdot 10^{-4}$	51
Mn^{2+}			$1.85 \cdot 10^{-4}$	32	$0.76 \cdot 10^{-4}$	47
Zn^{2+}			$1.85 \cdot 10^{-4}$	34	$0.76 \cdot 10^{-4}$	49
Cd^{2+}			$1.85 \cdot 10^{-4}$	36	$0.76 \cdot 10^{-4}$	52
Hg^{2+}				0		



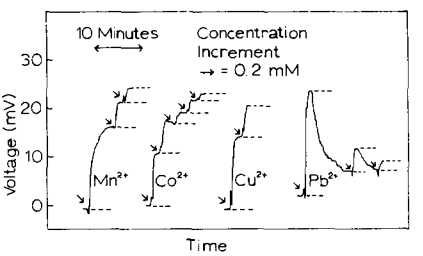


Fig. 3. Voltage traces for the alkaline earth and Group IIb chlorides. Buffer was 5 mM Tris, 5 mM NaCl (pH 7.0).

Fig. 4. Voltage traces for some transition metal chlorides. Lead was injected as the acetate. Buffer was 5 mM Tris, 5 mM NaCl (pH 7.0).

injected into the outer chamber but inhibited the ability of Zn^{2+} to induce a voltage. The voltage trace for lead shows the effect of precipitation from solution with concomitant loss of the voltage.

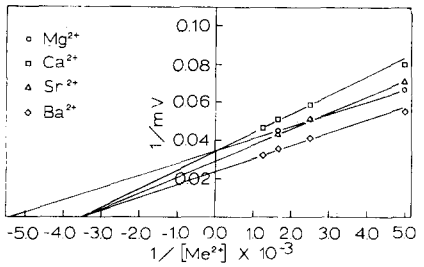


Fig. 5. Reciprocal voltage *versus* reciprocal alkaline earth ion concentration.

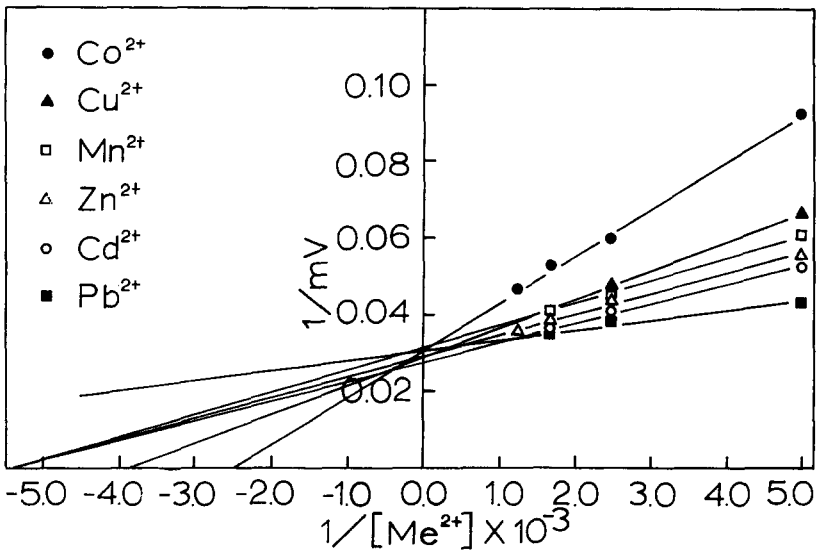


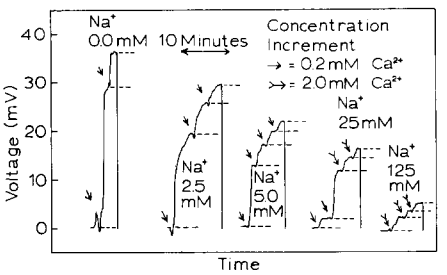
Fig. 6. Reciprocal voltage *versus* reciprocal Group IIb and transition metal ion concentration.

A comparison of the univalent *versus* the divalent cations reveals more than an order of magnitude greater dissociation of the alkali ions, $4 \cdot 10^{-3}$ M *versus* about $2 \cdot 10^{-4}$ M for the divalent cations. The voltage maxima for the univalent cations vary considerably but peak at about 71 mV for K^+ and Na^+ while the divalent cations, being uniform, gave values around 33 mV. It should be noted that the voltage maxima for the divalent cations would be higher and the dissociation constants smaller if the 5 mM NaCl were not present in the buffer. The uniformity of the voltage maxima for the divalent metals *versus* the variability of the monovalent cations suggests different mechanisms for the induction of the transmembrane potential.

(2) The effect of a second cation on the Ca^{2+}-induced voltage when located on both sides of the membrane

It was observed that the presence of NaCl in the buffers tended to repress the Ca^{2+}-induced transmembrane voltage. The repression effect was, therefore, studied

with various concentrations of the alkali cation chlorides present on both sides of the membrane at the same concentration. Fig. 7 shows the voltages for Ca^{2+} with various concentrations of NaCl present in the buffers. The double reciprocal plot is shown in Fig. 8.



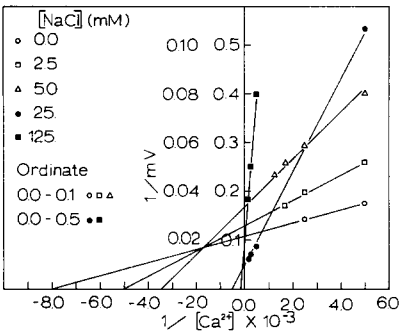


Fig. 7. Voltage traces for Ca^{2+} with the buffers containing 5 mM Tris (pH 7.0) and various NaCl concentrations.

Fig. 8. Reciprocal voltage *versus* reciprocal Ca^{2+} concentration with various concentrations of NaCl.

It is immediately apparent that NaCl can depress the Ca^{2+}-induced voltage and that the effect is increased by increasing the concentration of NaCl. The repression effect occurs both in the voltage maxima (decreasing with increasing alkali cation concentration) and the dissociation constants (increasing with increasing alkali cation concentration). The voltage traces and double reciprocal plots for Ca^{2+} with various concentrations of LiCl, KCl, or CsCl in the buffers appeared similar to those for NaCl and were therefore omitted. However, the values for the dissociation constants and voltage maxima for Ca^{2+} with various concentrations of all the alkali cations studied are presented in Table II.

TABLE II

DISSOCIATION CONSTANTS AND VOLTAGE MAXIMA FOR Ca^{2+} IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF THE ALKALI CATION CHLORIDES

Concn. (*mM*)	*LiCl*		*NaCl*		*KCl*		*CsCl*	
	$K \times 10^4$ (*M*)	*V* (*mV*)	$K \times 10^4$ (*M*)	*V* (*mV*)	$K \times 10^4$ (*M*)	*V* (*mV*)	$K \times 10^4$ (*M*)	*V* (*mV*)
0.0	—	—	1.23	48	—	—	—	—
2.5	2.3	50	2.5	40	2.6	46	2.5	36
5.0	3.9	44	2.9	33	3.9	40	3.6	29
10.0	3.1	33	—	—	—	—	5.3	21
25.0	16	29	17	21	14	28	15	15
125.0	—	—	77.0	13	77.0	10	—	—

In order to test the repression effect to see if it compared with the competitive binding model, the dissociation constants for Ca^{2+} were plotted as a function of the concentration of the alkali cation, an approach suggested by Eqn. 3. As is seen in Fig. 9 the points fall on a straight line. Using the dissociation constant for Ca^{2+} *plus*

the slope for each curve the dissociation constant for Ca^{2+} repression for the various monovalent cations can be determined. These values are listed in Table III along with the values obtained from single cation experiments for comparison.

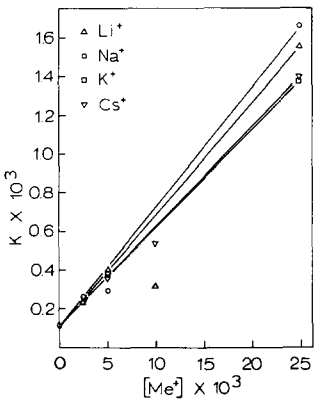


Fig. 9. Apparent dissociation constants *versus* alkali ion concentration in the buffers for Ca^{2+}-induced voltages.

(3) Correction of the divalent cation dissociation constants and voltage maxima

The dissociation constants listed for the divalent cations in the presence of 5 mM NaCl would be smaller and the voltage maxima greater if no NaCl was used in the buffers. If one assumes that all the divalent cations behave similar to Ca^{2+} with respect to the bimolecular lipid membrane potential, values of dissociation constants and voltage maxima can therefore be estimated for zero NaCl levels in the buffers.

The correction factor for Ca^{2+} from 5 mM NaCl to zero mM NaCl is (value in 0 mM NaCl)/(value in 5 mM NaCl). That is, the correction factor for the dissociation constants is $1.2 \cdot 10^{-4}/2.9 \cdot 10^{-4} = 0.41$. The factor for correcting the voltage maxima is $48/33 = 1.45$. Using these figures obtained for Ca^{2+} the corrected values for the various divalent cations are listed in the last two columns of Table I.

(4) The effect of pH on the Ca^{2+}-induced voltage

The effect of a variation in the pH of the buffers on the Ca^{2+}-induced voltage

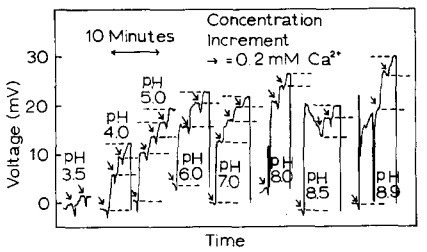
TABLE III

DISSOCIATION CONSTANTS, K_a, FOR THE ALKALI METALS DETERMINED FROM SINGLE CATION AND DOUBLE CATION EXPERIMENTS

Alkali metal	$K_a \times 10^3$ (M)	
	Alkali metal chloride (both sides) + $CaCl_2$ (one side)	*Alkali metal alone (from Table I)*
Li^+	2.14	4.07
Na^+	2.07	4.26
K^+	2.52	3.94
Cs^+	2.47	3.73

is shown in Fig. 10. The buffers ranged from pH 3.5 to 8.9 and care was taken to make the Na^+ concentration 5 mM in every buffer. The reciprocal plots of voltage and Ca^{2+} concentration are shown in Fig. 11. From the ordinate and abscissa intercepts the voltage maxima and dissociation constants were obtained and plotted as a function of pH as shown in Fig. 12. It can be seen that both the dissociation constant and voltage maxima show little variation between pH 4 and 8. Below pH 3.5 the voltage maximum is zero and above pH 8.5 displays an increase. The dissociation constant is remarkably constant above pH 5.5 but increases below that pH.

The effect of short circuiting the load resistor of the electrometer is seen in Fig. 10, pH 8.9. The voltage immediately drops to zero but rises back to its original value when the electrometer is reinserted into the circuit.



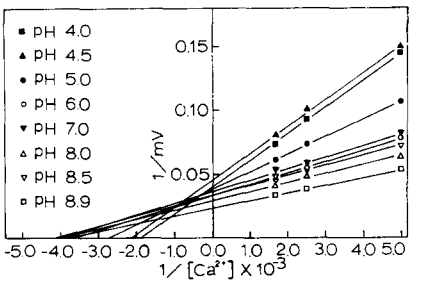
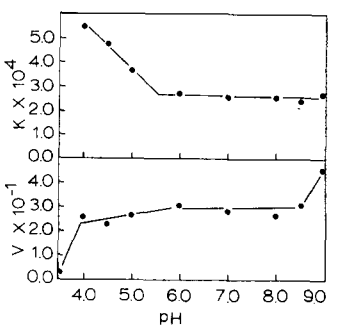


Fig. 10. Voltage traces for Ca^{2+} at various pH's. pH 3.5–5.0, 5 mM sodium acetate; pH 6.0, 2.5 mM Na_2HPO_4; pH 7.0–8.5, 5 mM Tris and 5 mM NaCl; pH 8.9, 5 mM glycine and 5 mM NaCl. All buffers were adjusted with HCl.

Fig. 11. Reciprocal voltage *versus* reciprocal Ca^{2+} concentration at various pH's.

(5) *The effect of EDTA and pH gradients on the Ca^{2+}-induced voltage*

The inability of Ca^{2+} to induce a transmembrane potential with EDTA present was tested and shown in Fig. 13. The effect of pH gradients on the membrane are also shown. It can be seen that EDTA sequesters the Ca^{2+}-induced voltage with a



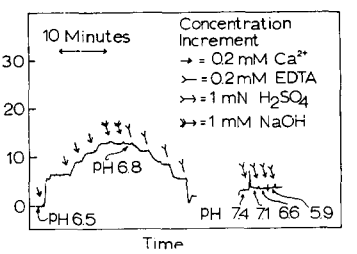


Fig. 12. Dissociation constants and voltage maxima for Ca^{2+} *versus* pH.

Fig. 13. Voltage traces for Ca^{2+} demonstrating effect of EDTA and the lack of an effect for a pH gradient. Buffers are: left trace: 10 mM KCl, 10 mM $NaHPO_4$ (pH 6.5); right trace: 1 mM Tris, 1 mM sodium acetate, 2 mM $CaCl_2$ (pH 7.4). Buffers adjusted with NaOH.

stoichiometry of Ca/EDTA: 1/1. This effect demonstrates that only the Ca^{2+} is involved in the membrane voltage. The production of H^+ concentrations across the membrane produce no apparent voltage.

DISCUSSION

The ability of the black or bimolecular lipid membrane to generate transmembrane potentials with a wide variety of cation salts has been demonstrated. The bimolecular lipid membrane used in the research was unmodified and consisted only of purified phospholipid from egg yolk and *n*-decane. No substances or agents were added to the lipid mixtures to enhance a voltage making ability.

Several authors have reported cation-induced transmembrane voltages with unmodified membranes. LÄUGER *et al.*[8] mention non-reproducible potentials with salts of sodium and potassium while HOPFER *et al.*[9] studied the effect extensively. Both authors and co-workers discuss the variation of voltage with the log of the ratio of the concentration of the active species on both sides of the membrane. BEAN AND SHEPHERD[17] showed voltages with Cd^{2+}, Zn^{2+} and Al^{2+}, but not with Ca^{2+}, Mg^{2+}, and Mn^{2+}. The current flow in the circuits of both LÄUGER *et al.*[8] and HOPFER *et al.*[9] were small or zero. This compared with the use of an input impedance of 10^{14} Ω for the electrometer used in this research. The important point is that the detector must have an internal impedance much higher than the membrane resistance or the membrane will be effectively short circuited and no voltage will appear.

The importance of using very fresh phospholipid preparations in the membrane cannot be overemphasized. LÄUGER *et al.*[8] were the only authors to use lipid mixtures freshly prepared every day. Their stock preparations were not allowed to be exposed to air because they filled the containers with glass beads. The stock preparations used in this research were gassed regularly with nitrogen, and the lipid mixtures, used for forming membranes, were made up 2 or 3 times daily. The buffers were also gassed routinely with nitrogen before making membranes.

Regardless of the type of cation used, the voltage induced across the membrane was always negative on the side containing the highest concentration of metal cation. The main feature observed in all the voltage traces in which cations were injected, regardless of whether monovalent or divalent, is that the increments for increased voltage decrease with equal incremental increases of cation concentration. If the voltage was only a function of the cation concentration in the buffer, the voltage increment should be linear with the concentration increment. The results suggest a Langmuir type of adsorption phenomenon. This contention is supported by the fact that plots of reciprocal voltage *versus* reciprocal cation concentration, result in straight lines with finite ordinate intercepts, at infinite cation concentration, indicating there exists a maximum voltage. Using the conditions for Langmuir-type adsorption, a voltage–adsorption equation has been derived for the case of cations adsorbing to the bimolecular lipid membrane. Assuming either adsorption or adsorption coupled with transport the same equation can be derived predicting the Langmuir type of voltage behavior with cation concentration.

The question of whether transport of cation across the bimolecular lipid membrane occurs cannot be answered by use of the voltage–adsorption equation because it can be derived with or without a transport step in the mechanism. More light may

be shed on this question, however, by using other available information. If adsorption were the only process inducing the voltage then all the alkali cations should have induced the same maximum voltage. This was not the case. The alkali cation voltage maxima showed large variations while the dissociation constants were essentially the same. This situation would argue for something other than simple adsorption when producing a transmembrane voltage. The fact that Hg^{2+} was incapable of inducing a voltage but was able to repress the voltage due to Zn^{2+} indicates that Hg^{2+} was possibly competitively adsorbing but, unlike Zn^{2+}, was unable to penetrate the membrane. Also the rise in voltage, after removing the short across the load resistor of the electrometer as seen in Fig. 10, pH 8.9, suggests that transport of cation occurs. If only adsorption were involved, the shorted circuit would allow cation to bind until the existence of equilibrium, according to the law of mass action. Replacement of the load resistor into the circuit should not cause any detection of voltage, if transport does not occur, because no more cation would be adsorbed. But a voltage is detected and it rises back to its original value. Furthermore, it may be argued that the detection of a potential difference across the membrane without a transport step would require the placement of the electrodes within the double layer of ions located on the membrane surface, 10–20 Å. Since the electrodes were located on the order of a centimeter from the membrane, a transport step in the mechanism seems probable.

The ability of EDTA to eliminate the Ca^{2+} voltage demonstrates that the Ca^{2+} is involved but unfortunately does not answer whether Ca^{2+} is transported since EDTA can be considered as a binding site in competition with that on the membrane but with a much smaller dissociation constant. It is assumed that the basic adsorption mechanism involved for Ca^{2+} is also present for the other divalent cations tested *plus* the monovalent cations. The repression of the Ca^{2+} induced voltage with the alkali ions suggests competitive binding to the same site on the membrane. The fact that the alkali cations have dissociation constants over an order of magnitude greater than Ca^{2+} and the other divalent cations would not necessarily indicate a different binding site since the selectivity coefficient for Ca^{2+} is about 20 times greater than Na^{+} when studied on a sulphonated polystyrene cation exchanger[18].

The values obtained for the dissociation constants for the various divalent cations (corrected for no NaCl in the buffers) are not out of line with other evidence regarding the binding of divalent cations to phospholipids. Equilibrium stability constants measured by HENDRICKSON AND FULLINGTON[19] for the binding of Ca^{2+}, Mg^{2+}, and Ni^{2+} to phospholipids in aqueous micellar dispersions, gave results comparable to those reported in this paper. Values on the order of $1 \cdot 10^{-4}$ M were found (reciprocal of the equilibrium stability constants) for the case of phosphotidylserine and triphosphoinositide when studying the binding of Ca^{2+} and Mg^{2+}. Ni^{2+} gave somewhat smaller values.

The structure on the membrane that is the most likely site at which Ca^{2+} binds may be the phosphate group on the phospholipids. Evidence supporting this contention stems from the effect of varying the pH below the pK of ionization of the phosphate group, thereby eliminating the ability of Ca^{2+} to bind to the membrane. The effect of pH on the binding of Ca^{2+} to monolayers of various phospholipids as studied by ROJA AND TOBIAS[20] showed that Ca^{2+} was uniformly bound to phosphatidylserine and phosphatidylethanolamine between pH's of about 4 and 8, dropping off to zero below pH's of about 3 and increasing above pH 8. Similar results for

monolayers were also obtained by PAPAHADJOPOULOS[21]. BLAUSTEIN[22] studying the partition of Ca^{2+} from an aqueous phase to a chloroform–methanol–phospholipid phase demonstrated a similar pH effect.

A possible mechanism for the transport of Ca^{2+} or other divalent cations through the bimolecular lipid membrane could involve the neutralization of charge both on the cation and the phospholipids as pictured by SHAW AND SCHULMAN[23]. The penetration into the hydrophobic environment by a charged species such as Ca^{2+} or $-HPO_2^{2-}$ in the phospholipids would be small. However, the combination of the two, which would neutralize the charge would greatly facilitate penetration and transport. The mechanism would also explain the similarity of most of the divalent cations with respect to both the dissociation constant and voltage maxima. A change in ionic radii for the divalent cations would not be enough to significantly effect the size of the metal–phospholipid complex. The total bulk of the complex would vary only slightly with the result that both dissociation constants and voltage maximum would be similar.

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REFERENCES

1 P. MUELLER, D. O. RUDIN, H. TI TIEN AND W. C. WESCOTT, in J. F. DANIELLI, K. G. A. PANHURST AND A. C. RIDDIFORD, *Recent Progress in Surface Science*, Academic Press, New York, 1964, p. 379–393.
2 P. MUELLER, D. O. RUDIN, H. TI TIEN AND W. C. WESCOTT, *J. Phys. Chem.*, 67 (1963) 534.
3 L. ROTHFIELD AND A. FINKELSTEIN, *Ann. Rev. Biochem.*, (1968) 463.
4 H. T. TIEN AND A. L. DIANA, *Chemistry and Physics of Lipids*, 2 (1968) 55.
5 P. MUELLER AND D. O. RUDIN, *Biochem. Biophys. Res. Commun.*, 26 (1967) 398.
6 A. A. LEV AND E. BUZHINSKY, *Tsitologiya*, 1 (1967) 102.
7 U. HOPFER, A. L. LEHNINGER AND T. E. THOMPSON, *Proc. Natl. Acad. Sci. U.S.*, 59 (1968) 484.
8 P. LÄUGER, W. LESSLAUER, E. MARTI AND J. RICHTER, *Biochim. Biophys. Acta*, 135 (1967) 20.
9 U. HOPFER, A. L. LEHNINGER AND W. J. LENNARZ, *J. Membrane Biol.*, 2 (1970) 41.
10 M. C. PANGBORN, *J. Biol. Chem.*, 188 (1951) 471.
11 P. G. ROUGHAN AND R. D. BATT, *Anal. Biochem.*, 22 (1968) 74.
12 C. LONG, *Biochemist's Handbook*, Van Nostrand, Princeton, N.J., 1961, p. 763.
13 A. ROTHSTEIN AND J. VAN STEVENINCK, *Biol. Membranes: Recent Progr., N.Y. Acad. Sci.*, 137 (1966) 606.
14 C. HUANG, L. WHEELDON AND T. E. THOMPSON, *J. Mol. Biol.*, 8 (1964) 148.
15 I. LANGMUIR, *J. Am. Chem. Soc.*, 38 (1916) 2221.
16 M. DIXON AND E. C. WEBB, *Enzymes*, Academic Press, New York, 1958, p. 22.
17 R. C. BEAN AND W. C. SHEPHERD, *150th Meeting Am. Chem. Soc., Atlantic City, N. J.*, Spaulding-Moss, Boston, 1965, p. 19c.
18 J. A. KITCHENER, *Ion Exchange Resins*, Wiley, New York, 1957, p. 23.
19 H. S. HENDRICKSON AND J. G. FULLINGTON, *Biochemistry*, 4 (1965) 1599.
20 E. ROJA AND J. M. TOBIAS, *Biochim. Biophys. Acta*, 94 (1965) 394.
21 D. PAPAHADJOPOULOS, *Biochim. Biophys. Acta*, 94 (1965) 394.
22 M. P. BLAUSTEIN, *Biochim. Biophys. Acta*, 135 (1967) 65.
23 D. O. SHAW AND J. H. SCHULMAN, *J. Lipid Res.*, 8 (1967) 227.