Ion Transport Through Excitability-Inducing Material (EIM) Channels in Lipid Bilayer Membranes

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ABSTRACT Two different methods were used to determine the relative permeability and the voltage-dependent conductance of several different cations in excitability-inducing material (EIM)–doped lipid bilayers. In one method, the conductances of individual channels were measured for Li, Na, K, Cs, NH_4, and Ca, and in the other method biionic potentials of a membrane with many channels were measured for Li, Na, K, Cs, and Rb. The experimental results for the two methods are in agreement. The relative permeabilities are proportional to the ionic mobilities in free aqueous solution. The voltage dependence of the conductance is the same for all cations measured.

INTRODUCTION

EIM (excitability-inducing material) is the name given by Mueller and Rudin (1963) to the protein material of bacterial origin which induces voltage-dependent ion conductance in lipid bilayer membranes. The EIM-doped bilayer possesses a negative differential conductance (Mueller and Rudin, 1968) similar to that exhibited by the potassium pathway of nerve axons in high external potassium solutions (Ehrenstein and Gilbert, 1966).

When very small amounts of EIM are added to the solution bathing a lipid bilayer membrane, the induced conductance develops by discrete steps (Bean et al., 1969). These steps arise from the formation of individual ionic channels. Voltage clamp experiments on oxidized cholesterol membranes with small numbers of EIM channels demonstrate that the individual channels undergo transitions between two states of different conductance. The fraction of time that a single channel is in the high-conductance state is the same function of voltage as the voltage-dependent conductance of the many-channeled membrane (Ehrenstein et al., 1970). Thus the negative differential conductance of the EIM-doped bilayer has its origin in the

voltage-dependent "opening" and "closing" of individual conducting channels. The EIM-doped bilayer is therefore a system in which an important property of excitable membranes—voltage-dependent conductance—is localized in the behavior of individual discrete conducting sites.

The purpose of this paper is to examine the ion transport properties of these individual EIM channels. We have measured the amplitudes of the current steps of single EIM channels in the presence of various ionic solutions in order to determine the sequence of relative permeabilities of the alkali cations. In addition, we determined these relative permeabilities independently by measuring biionic potentials across membranes doped with larger amounts of EIM.

The measured relative permeabilities are useful in the determination of the structure of EIM channels. The experiments also test whether the channel gating process is influenced by the type of cation in solution. A preliminary report of these experiments was presented at the 1971 Biophysical Society Meetings (Lecar et al., 1971).

METHODS

All of the experiments reported in this paper were performed on oxidized cholesterol membranes, which are stable for 1–2 hr at room temperature. The oxidized cholesterol used to form the membranes was prepared by the method of Tien et al. (1966). The stock solution of EIM was prepared according to the method of Kushnir (1968). The complete procedure as employed here was described in a previous paper (Ehrenstein et al., 1970).

The experimental arrangement for forming and studying the lipid bilayer membranes is shown in Fig. 1. The design of the apparatus follows that of Mueller et al. (1964) with minor modifications. Membranes are formed from a solution of 20 mg of oxidized cholesterol in 1 ml of decane. The solution is brushed across a hole in the wall of the inner cup shown in the figure. The cup is made of Teflon (volume, 5 ml) and the hole is 1 mm in diameter. All of our experiments were performed at temperatures between 25° and 26°C. At this temperature, membrane formation occurs within approximately 1 min.

Fig. 1 also shows the circuit for measuring and recording the electrical properties of the membrane. Currents and voltages were introduced through calomel electrodes connected to the solution chamber through 0.1 M KCl-agar bridges. The variable resistor R_A is the ammeter resistance. In all the measurements, the direction of positive current flow is defined as cation flow into the compartment containing EIM, which is the inner compartment in these experiments. The amplifier S, the output of which is the sum of the various pulses present at the summing junction, is the effective voltage source for the membrane. Because of the high resistance of the membrane (10^9–10^{10} ohms), the potential across the membrane is essentially equal to the source potential, and "voltage clamp" conditions are attained without external feedback. Biionic potentials were measured using a Keithley electrometer (Keithley Instru-

ments, Inc., Cleveland, Ohio). For DC measurements the voltage source S was switched to open circuit.

Membranes having small numbers of EIM channels are obtained by adding 10 μl of a 10% dilution of the stock EIM solution to the inner compartment. The number of channels produced in this way varies from membrane to membrane. However, by varying the position of the pipette used to add the solution and varying the dilution, membranes having one to five channels could be produced.

For measurement of the membrane potential developed by a concentration gradient of two different cations, the concentration of one cation is kept constant at 30–50 mM in the compartment containing EIM, while the concentration of the cation in the other compartment is gradually increased.

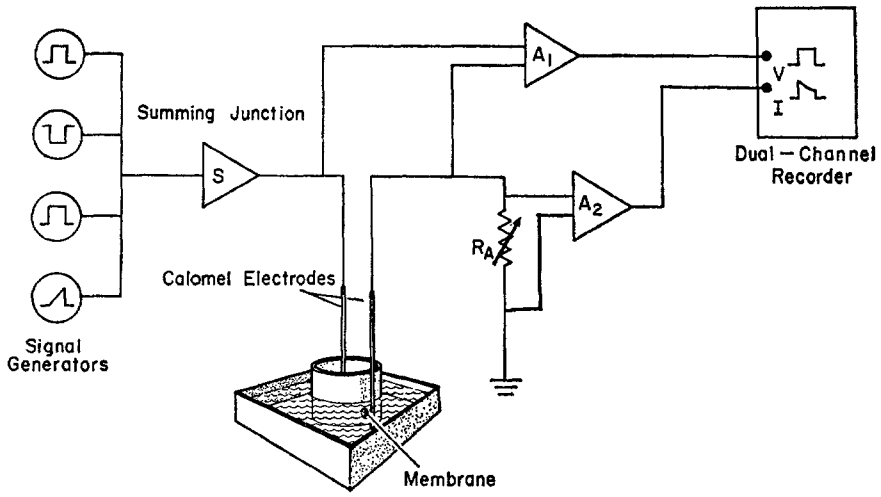

FIGURE 1. Experimental arrangement for studying electrical properties of bilayers.

Biionic potentials are measured as follows. The membranes are formed in a solution of 2 mM tris(hydroxymethyl)amino methane (Tris)–chloride buffer (pH 7). After the membrane is formed in this solution, the concentration of the solution in the inner compartment is raised to 30 mM by adding a small sample of a 3 M solution of the salt to be tested. The same procedure is then followed with a different ionic solution for the outer compartment. Under these conditions, the membrane potential remains close to zero. Sufficient EIM is then added to reduce the membrane resistance by two to three orders of magnitude, and the membrane potential reaches steady state within a few minutes.

RESULTS

Channel Conductances

Fig. 2 shows the trains of steplike current fluctuations obtained in 0.1 M KCl when a steady voltage is applied across a membrane with a small number of EIM channels. Typical step patterns are seen for different membranes

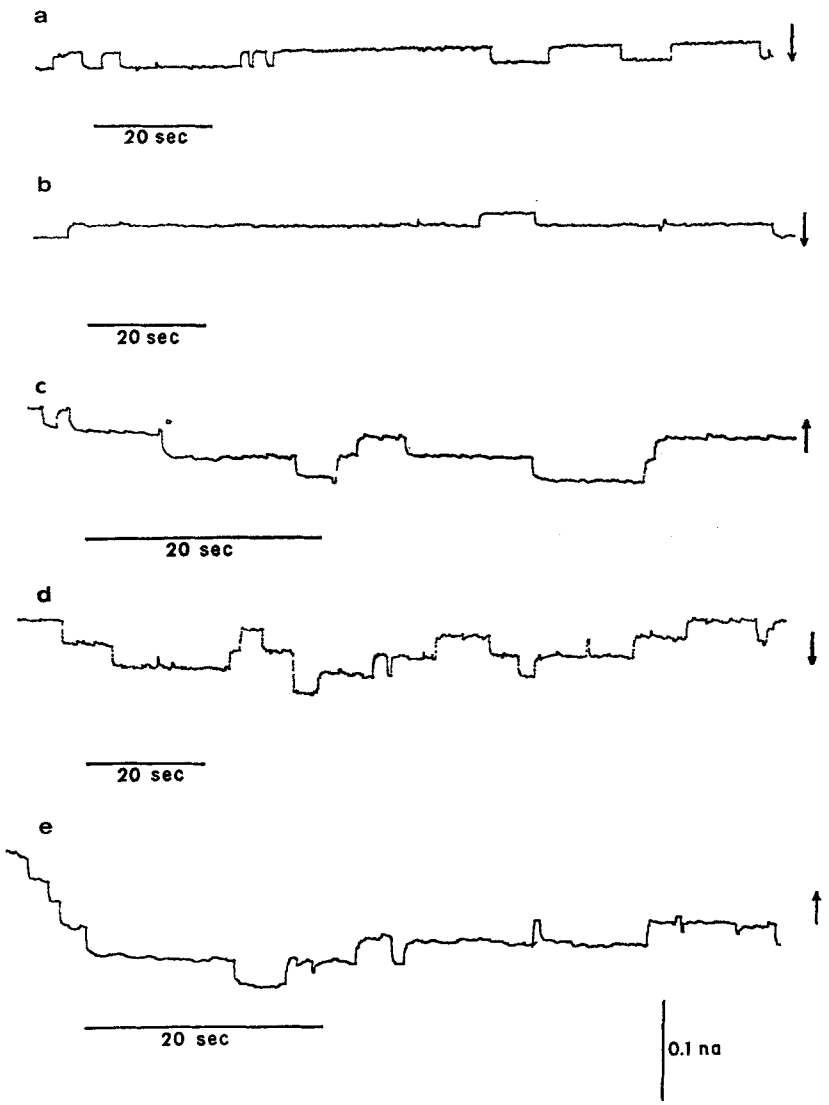

FIGURE 2. Typical segments from records of discrete current steps. These records were made on membranes in 0.1 M KCl. (*a*), Single-channel membrane at 51 mv. (*b*), Two-channel membrane at 50 mv. (*c*), Three-channel membrane at 60 mv. (*d*), Four-channel membrane at 75 mv. (*e*), Five-channel membrane at 70 mv. The arrow in each case indicates the direction of increasing current.

with one, two, three, four, and five channels. Fig. 3 shows the steps of different amplitude obtained at a given voltage for membranes with equal concentrations of KCl and LiCl. The variation of step amplitude with changes in the ionic composition indicates that the channel conductance is different for each ion. Fig. 4 shows several single-channel difference current-voltage

curves (difference between "open" and "closed" channel) obtained with different cations by plotting the mean amplitude of the current steps as a function of applied voltage. The current-voltage plots are linear, with the slope of the line giving the single-channel conductance step in a particular

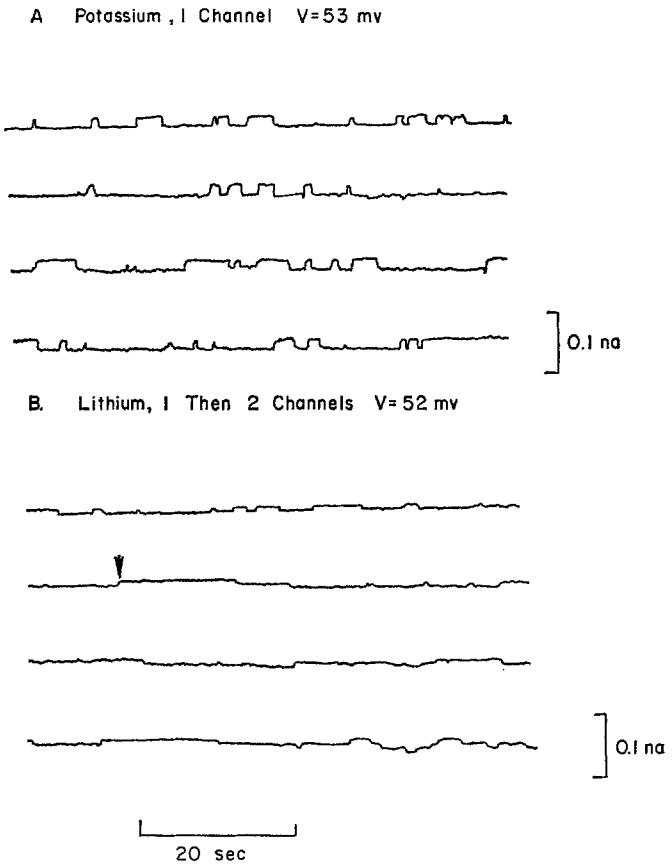

FIGURE 3. Comparison of curent steps with different ions. (A), Single-channel membrane in 0.1 M KCl. (B), Single-channel membrane in 0.1 M LiCl. The arrow indicates the first appearance of a second channel. For each solution, the four traces shown are consecutive segments of one record.

salt solution. The conductance steps measured for several cations are listed in Table I.

In order to test whether changes in ionic environment alter the average fraction of time that a channel spends in the open state at a given voltage, we obtained the statistics of channel transitions from records such as those of Fig. 2. For a membrane with N channels, the average fraction of time that a

single channel is open at a particular voltage is given by

$$f(V) = (1/NT) \sum_{n=0}^{N} n \cdot t_n , \qquad (1)$$

where T is the total time of the voltage record, n is one of the possible values

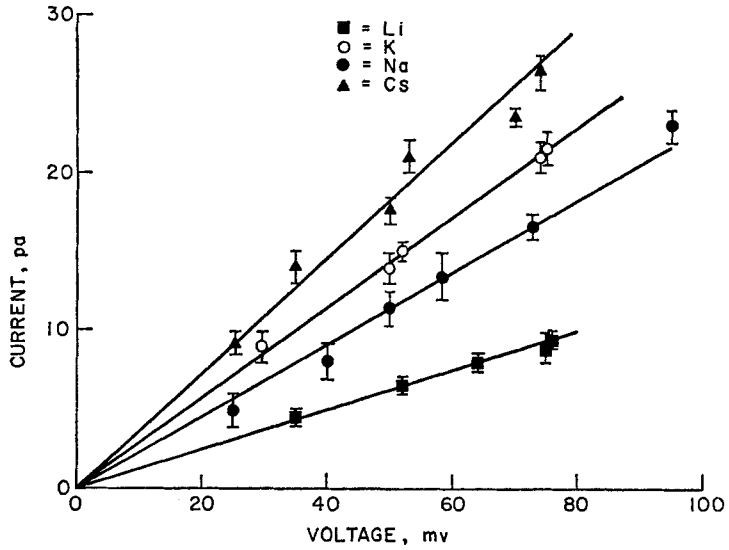

FIGURE 4. Difference current-voltage curves for membranes with one to three EIM channels. The symbols used for the experimental points denote the different cations used: ■, Li; ○, K; ●, Na; ▲, Cs. The vertical bars represent the experimental variance of the step heights. The solutions are 0.1 M chloride salts of the indicated cation.

TABLE I

SINGLE-CHANNEL CONDUCTANCE STEPS

Ion	Conductance	Number of steps measured
	10^{-10} mho	
Li	1.3±0.1	230
Na	2.6±0.2	114
K	3.1±0.4	266
Cs	3.6±0.2	147
NH_4	3.0±0.4	72
Ca	2.2±0.1	99

for the number of open channels (i.e., $n = 0, 1, 2, 3, \cdots, N$), and t_n is the amount of time that n channels are open. Values of $f(V)$ obtained in this way are plotted in Fig. 5 *a*. There is no significant difference in the function $f(V)$ for the different cations. The experimental points of the figure were obtained for membranes with different numbers of channels

(one to five channels), and it can be seen that the shape of the $f(V)$ curve does not depend on the number of EIM channels.

For a membrane with many channels, the property corresponding to $f(V)$ is the voltage-dependent conductance $g(V)$. If the channels are inde-

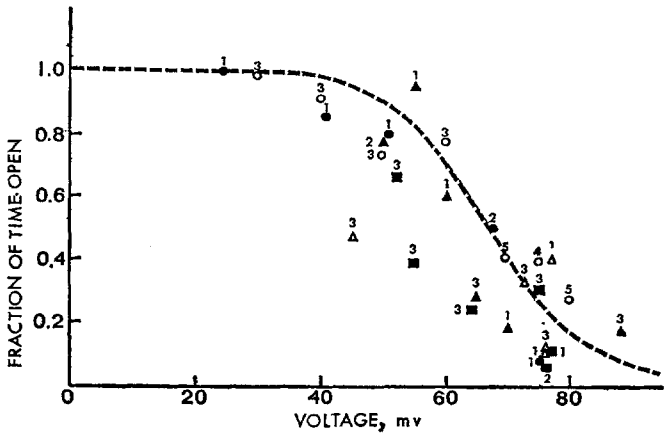

FIGURE 5 *a*

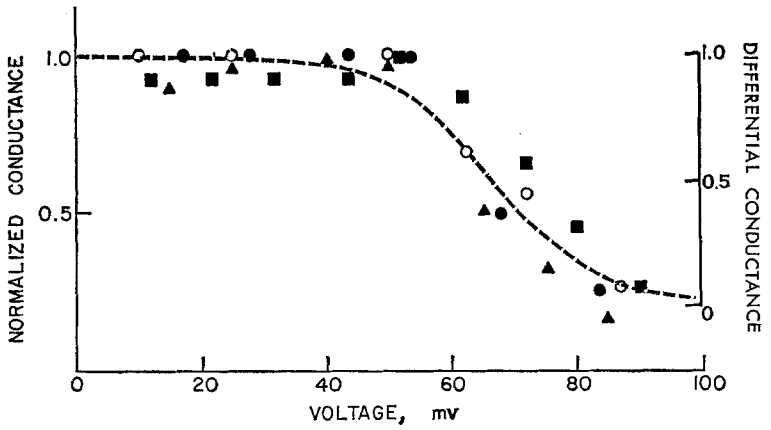

FIGURE 5 *b*

FIGURE 5 *a*. Fraction of time that a channel is in the open conducting state as a function of voltage. Data are taken from experiments with one, two, three, four, or five channels, as indicated by the number next to each experimental point. The symbols used for the experimental points denote the different cations used: ■, Li; ○, K; ●, NH_4; ▲, Cs; △, Na. The solutions are 0.1 M chloride salts of the indicated cation. Dashed curve is equilibrium distribution of number of open channels for a two-state model.

FIGURE 5 *b*. Normalized voltage-dependent conductance of many-channel membrane measured with different ions. The figure illustrates the invariance of the steady-state fraction of open channels for the various monovalent cations. Dashed curve and symbols for cations are the same as in Fig. 5 *a*.

pendent, then

$$g(V) = Ng_{\Delta}f(V), \tag{2}$$

where g_{Δ} is the difference conductance of a single channel (the difference in conductance between an open and closed channel). Fig. 5 *b* shows the normalized voltage-dependent conductance (i.e., $g(V)/g_{max}$) for membranes

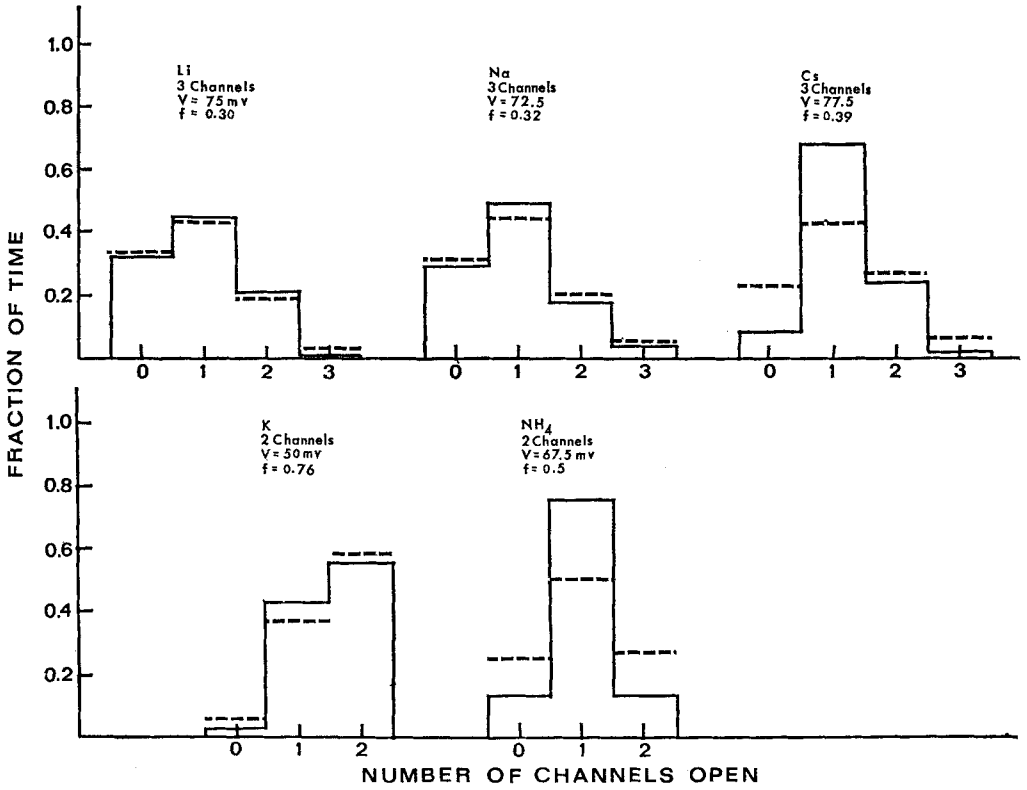

FIGURE 6. Typical histograms of the fraction of time a specific number of channels remains open for various membranes at different voltages. The dashed histograms show theoretical values for a model of independent two-state channels. The values of f shown in the figure are experimental values determined from equation 1 (see Ehrenstein et al., 1970).

with many EIM channels and equivalent solutions of different alkali cations. The shape of the conductance curve is seen to be the same as $f(V)$ and independent of the cation involved. Thus, for any number of conducting channels, the probability of a channel being open or closed as a function of voltage is independent of the ion being transported. The dashed curve shown in both Figs. 5 *a* and 5 *b* is the equilibrium distribution of the number of open channels for a simple model of two-state channels; this curve is taken directly

from the paper of Ehrenstein et al. (1970), without any adjustment of parameters.

The times that a membrane with a small number of channels spends in a state with 0, 1, 2, 3 · · · channels open can be displayed as a histogram. Previously, histograms for EIM transitions in the presence of KCl were shown to obey the binomial distributions expected for independent two-state channels (Ehrenstein et al., 1970). Fig. 6 shows histograms taken at various voltages in the presence of different cations. The figure demonstrates that the channel statistics obey a binomial distribution with a probability parameter that is a function of voltage alone, independent of the cation.

The large amount of data collected in the process of measuring the single-

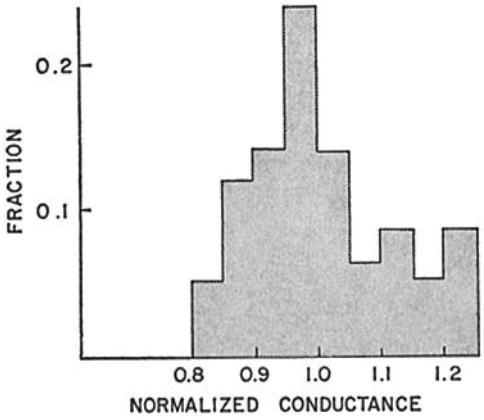

FIGURE 7. Distribution of channel conductances for 92 individual EIM channels. Conductances measured with different ionic solutions are normalized to the mean conductance measured with that solution.

channel conductances allows us to answer the question, how uniform are the individual EIM conducting units. Previous work (Bean et al., 1969; Ehrenstein et al., 1970) showed some spread in the channel conductances, but there were not enough data to draw quantitative conclusions. In the present work, data were obtained on 92 EIM channels and the conductances observed can all be displayed on the same histogram when they are normalized to the average conductance for a given cation. This is shown in Fig. 7, in which it is seen that the spread in normalized channel conductance is less than $\pm 20\%$. This dispersion in channel conductance is larger than that expected from the amplitude variations in any single-channel experiment (which range from 5 to 10%). Hence, the histogram shows a relatively small nonuniformity of individual conducting channels. More important, the data indicate that EIM channels are relatively homogeneous conducting units rather than random-size conducting domains.

Biionic Potentials

A more conventional measure of ionic selectivity than the conductance jumps is the biionic potential. We determined biionic potentials across membranes containing larger amounts of EIM (of the order of 100 channels), and made corrections for liquid-junction potentials using the Planck formula (MacInnes, 1961). The results are summarized in Table II.

For perfectly cation-selective channels, the relative permeabilities can be calculated from the steady-state membrane potentials by using the equation (Helfferich, 1962)

$$V = \frac{RT}{F} \ln \frac{P_a[a]}{P_b[b]}, \tag{3}$$

where P_a and P_b are the cation permeabilities, V is the potential, a and b

TABLE II

BIIONIC POTENTIAL MEASUREMENTS

Solution	Measured potential	Biionic potential corrected for liquid junctions	Biionic permeability ratio
	mv	*mv*	
KCl-LiCl	15.1±1.3	21.7	2.4
NaCl-LiCl	9.4±0.6	11.8	1.6
KCl-NaCl	9.4±1.0	13.7	1.7
KCl-RbCl	0.8±1.2	0.1	1.0
KCl-CsCl	−0.9±1.0	−1.5	0.94

are the activities of the ions involved, and R, T, and F have their usual meanings. As long as the membrane passes cations only, equation 3 can be derived by direct integration of the electrodiffusion equation, without any further assumptions. Permeability ratios obtained from equation 3 for the special case $[a] = [b]$ (biionic conditions) are listed in Table II.

A test for selectivity between cations and anions is the concentration potential. With the unmodified membranes, no potential difference was observed even with 40:1 ratios of salt concentration. Mueller and Rudin (1968) demonstrated that for EIM-treated lipid membranes the potential as a function of concentration ratio for KCl had a slope approximately equal to 58 mv/decade, the thermodynamic value expected when anions are completely excluded from the membrane. This limiting value is also expected for the case of dissimilar cations on both sides of the membrane, as can be seen from equation 3. Fig. 8 shows the potentials measured when the concentration of NaCl on one side of the membrane is fixed and the concentration of LiCl is varied. The dashed line calculated from equation 3 (for com-

plete anion exclusion) has a slope of 58 mv/decade, independent of the relative permeabilities of the cations. The observed slope in Fig. 8 is 59 ± 2 mv/decade, and in another similar experiment the observed slope was 56 ± 4 mv. The curvature seen in Fig. 8 occurs at high concentration ratios. This curvature may be related to fixed charge in the EIM channel, or may merely be a result of the low salt concentrations on one side of the membrane needed to obtain these high concentration ratios. In experiments in which the concentration of KCl on one side of the membrane was fixed and the concentration of NaCl was varied, the average observed slope for three experiments was 50 ± 3 mv, and the curvature at high concentration ratios was similar to that in Fig. 8.

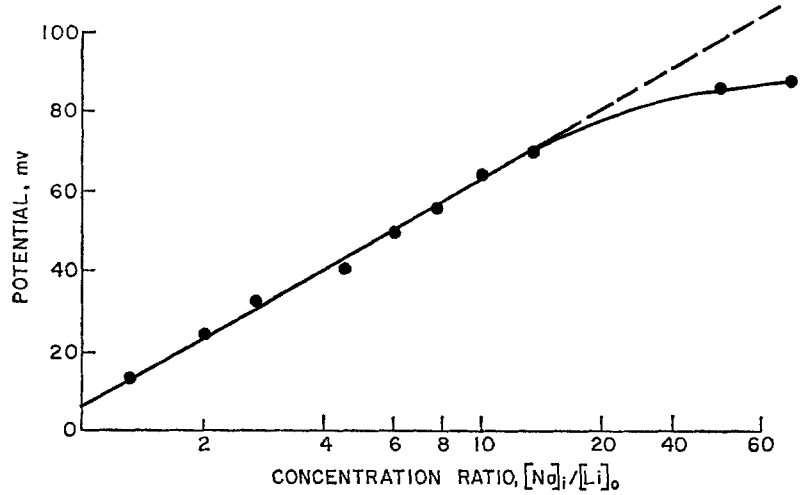

FIGURE 8. Steady-state membrane potential for concentration gradients of two different cations on either side of the membrane. The abscissa is the ratio of Na concentration in the inner compartment to Li concentration in the outer compartment. The anion was Cl. The dashed line is calculated under the assumption of complete anion exclusion.

DISCUSSION

It has been shown that the voltage-dependent conductance of EIM is caused by the opening and closing of individual channels (Ehrenstein et al., 1970). Figs. 5 *a* and 5 *b* illustrate the identity of the voltage dependence of the single-channel transition mechanism $f(V)$ and the many-channel conductance $g(V)$ for different cations. These figures and the histograms of Fig. 6 demonstrate that EIM channels act independently and that the mechanism of opening and closing channels does not depend on the cation transported.

The measurements of the single-channel conductance steps and the biionic potentials provide two independent means of determining the ionic selectivity of the EIM channels. In Fig. 9, we compare the relative permeabilities

of the alkali cations obtained by the two methods. Within experimental error, the relative permeability values obtained by the two methods are in agreement. This is further confirmation that the conductance steps represent changes in current through discrete channels in the membrane, and that the conductance of EIM-doped bilayers arises from the flow of ions through these discrete channels.

The relative permeabilities are very close to the ratios of mobilities of the ions in free solution. This might suggest that the ions travel through the

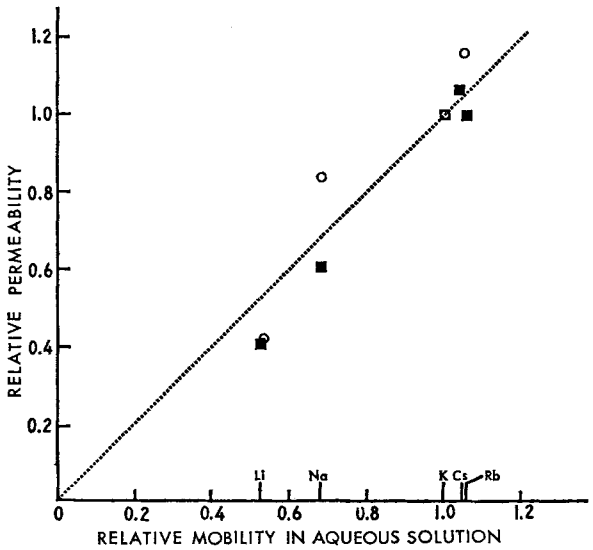

FIGURE 9. Measured values of relative permeability for Li, Na, K, Cs, and Rb as a function of relative mobility in aqueous solution for these cations. All values referred to potassium ion. Relative mobilities are from Robinson and Stokes (1959). ○, relative permeability determined from conductances of single channels. ■, relative permeability determined from biionic potentials. The dashed line represents perfect correlation between relative permeability through the membrane and relative mobility in aqueous solution.

EIM channel with relatively unperturbed hydrations. More likely, the EIM channel does not itself discriminate among cations, and the relative rates of transport are determined primarily by the frequency of collisions between ions and the mouth of the channel. Since these collisions occur in the aqueous medium, the free-solution mobilities would enter as a multiplicative factor in a determination of the rate of transport, provided that ions do not bind strongly to the channel. The concentration potentials shown in Fig. 8 (also Mueller and Rudin, 1968) indicate a high degree of selectivity for cations in preference to anions. The combination of preference for cations and little intercationic selectivity is similar to the behavior of wide-pore fixed–negative

site ion exchangers (Eisenman et al., 1967). The wide-pore behavior implies that the ions see a weak electric field caused by the membrane fixed charges. This can be either because the negatively charged groups are at the surface of a large pore or because the fixed charge is located within the channel structure but remote from the transport path.

If the observed selectivity is, indeed, the result of a negative fixed charge, it is of interest to estimate the charge density. A rough estimate of the fixed charge density within a channel needed to give the observed concentration-potential slope (more than 53 mv/decade) can be obtained from the theory of fixed charge membranes (Teorell, 1953). This theory gives the membrane potential as the sum of Donnan potentials at the membrane-solution interfaces and a diffusion potential across the membrane (equation 19:2 of Teorell, 1953). These potentials depend upon the fixed charge density (equations 8:2 and 22:1, 2, 23, and 24 of Teorell, 1953). By substituting various values for the fixed charge density, we found that the minimum value consistent with the observed concentration-potential slopes is about 200 mM. Experimental evidence for a fixed charge density of this magnitude for EIM-treated bilayers has recently been obtained by Dr. R. C. Bean (personal communication). He examined the relation between ionic concentration and channel conductance, and found that there is a linear relation for concentrations larger than about 200 mM, but that conductance tends to reach a lower limit at about 100 mM.

The conductance of an open EIM channel (the residual conductance of a closed channel plus the difference conductance) is approximately 4×10^{-10} mho in 0.1 M KCl. The diameter of this channel can be estimated on the assumption that the channel is a cylindrical pore filled with the ambient electrolyte. For a membrane thickness of 40 A (Tien et al., 1966) the pore diameter is approximately 16 A. Such a large pore might be expected to allow ions to permeate with their free-solution mobilities provided that the state of the polar medium of the pore allows easy reorientation of ion hydration shells. However, a large conductance per channel does not necessarily imply wide-pore behavior in all cases. The membrane could be pinched in at the channel site, for example, or the high electric field across the membrane might drive ions through the channel more rapidly than expected from the free solution mobilities. In fact, estimates of the order of 10^{-10} mho have been given for the sodium conduction sites of the axon membrane (Hille, 1970), and these sites exhibit narrow-pore selectivity (Chandler and Meves, 1965; Hille, 1971).

By combining our rough estimates of 16 A for the pore diameter and 200 mM for the effective fixed charge density within the pore, we can obtain an estimate of the number of fixed charges contained in a single channel. This value is approximately one electronic charge per channel. All of our results

are consistent with a picture of the EIM channel as a relatively large, water-filled pore, lined with fixed negative charge.

We wish to thank Mr. Mark Holbert for his assistance in these experiments and Dr. Kenneth Cole or his helpful criticism of the manuscript.

Received for publication 30 December 1971.

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