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The Effects of the Macrotetralide Actin Antibiotics on the Electrical Properties of Phospholipid Bilayer Membranes*

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Summary. This paper, the last in a series of three, characterizes the electrical properties of phospholipid bilayer membranes exposed to aqueous solutions containing nonactin, monactin, dinactin, and trinactin and Li^+, Na^+, K^+, Rb^+, Cs^+, and NH_4^+ ions. Not only are both the membrane resistance at zero current and the membrane potential at zero current found to depend on the aqueous concentrations of antibiotic and ions in the manner expected from the theory of the first paper, but also these measurements are demonstrated to be related to each other in the manner required by this theory for "neutral carriers". To verify that these antibiotics indeed are free to move as carriers of cations, cholesterol was added to the lipid to increase the "viscosity" of the interior of the membrane. Cholesterol decreased by several orders of magnitude the ability of the macrotetralide antibiotics to lower the membrane resistance; nevertheless, the permeability ratios and conductance ratios remained exactly the same as in cholesterol-free membranes. These findings are expected for the "carrier" mechanism postulated in the first paper and serve to verify it. Lastly, the observed effects of nonactin, monactin, dinactin, and trinactin on bilayers are compared with those predicted in the preceding paper from the salt-extraction equilibrium constants measured there; and a close agreement is found. These results show that the theory of the first paper satisfactorily predicts the effects of the macrotetralide actin antibiotics on the electrical properties of phospholipid bilayer membranes, using only the thermodynamic constants measured in the second paper. It therefore seems reasonable to conclude that these antibiotics produce their characteristic effects on membranes by solubilizing cations therein as mobile positively charged complexes.

Starting from the postulate that molecules such as the neutral macrocyclic antibiotics solubilize cations in the interior of a phospholipid membrane as mobile positively charged complexes, the preceding two papers

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(Ciani, Eisenman & Szabo, 1969; Eisenman, Ciani & Szabo, 1969) of this series of three, referred to hereafter as I and II, have deduced the theoretically expected effects of these "carrier" molecules on the electrical properties of thin (e.g., 60 A) membranes as well as on the equilibrium extraction of cations and anions into bulk organic solvents. It was shown in papers I and II that from this postulate particular interrelationships are expected between the effects of these molecules on membrane resistance and membrane potential, and, moreover, that these membrane properties should be quantitatively relatable to the equilibrium constants measurable for the salt extraction into bulk organic solvents. These papers have also shown not only how an appropriate organic solvent can serve as a simplified model for the hydrocarbon interior of the phospholipid bilayer membrane, but also, and more particularly, how certain properties of a phospholipid membrane exposed to such antibiotics can be predicted from studies on any convenient arbitrarily chosen solvent provided only that the complexes are "isosteric" (i.e., have essentially the same size and shape regardless of the particular cation species sequestered).

The present paper characterizes the effects of the macrotetralide actin antibiotics (nonactin, monactin, dinactin, and trinactin) on the electrical properties of lecithin and lecithin-cholesterol bilayer membranes exposed to aqueous solutions containing Li^+, Na^+, K^+, Rb^+, Cs^+, and NH_4^+ ions. It is shown that the membrane conductance at zero current and the membrane potential at zero current depend on the aqueous concentrations of antibiotic and ions in the manner expected from the theory of paper I, and also that these measurements are interrelated in the theoretically predicted manner. Moreover, when the "fluidity" of the lipid interior is altered by adding cholesterol to the lipid, the observed effects on membrane resistance and potential are found to be exactly those expected for the "carrier" mechanism postulated in paper I. Lastly, the quantitative predictions of papers I and II for the effects on bilayers of nonactin, monactin, dinactin, and trinactin are tested and verified. These results all support the postulate that the macrotetralide actins produce their characteristic effects on bilayer membranes by acting as mobile carriers of cations.

Theoretical Expectations

Membrane Potential and Membrane Conductance

For a membrane of the dimensions of a phospholipid bilayer (i.e., one whose thickness is less than the apparent Debye length within the hydrocarbon phase), it was shown in paper I that the cation-antibiotic complexes,

IS^+, are the major charge-carrying species within the membrane, being present as an excess space charge. These complexes therefore determine the electrical properties of the membrane. An explicit expression for the membrane potential for mixtures of cations was derived by integrating the Nernst-Planck flux equation across the membrane for the IS^+ complexes and expressing the concentrations of these in terms of the (given) aqueous concentrations of antibiotic and ions [*see* Eq. (19, I)].[1] When the concentration of antibiotic-cation complexes in the aqueous solutions is negligible (as expected for the macrotetralide actins), and for the simple experimental situation to be studied here where the antibiotic concentration is the same on both sides of the membrane, this equation reduces to [*see* Eq. (22, I)]:

$$V_0 = \frac{RT}{F} \ln \frac{a_i' + \beta a_j'}{a_i'' + \beta a_j''}. \tag{1}$$

Eq. (1) expresses the measurable membrane potential, V_0 (i.e., the potential difference between the aqueous solutions), in terms of the activities, a_i', a_j', a_i'', a_j'', of the ions, I^+ and J^+, in the aqueous solutions on the two sides (′) and (″) of the membrane.

The constant β of Eq. (1), expected from the theory of paper I to be characteristic of the relative effects of I^+ and J^+ for a given antibiotic and membrane composition, is recognizable as formally equivalent to the permeability ratio of the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin & Katz, 1949), and is defined in terms of the ratio of the mobilities, u_{js}^* and u_{is}^*, of the JS^+ and IS^+ complexes in the membrane and the ratios of the equilibrium constants of reactions (1, I) and (4, I) as:

$$\beta = \frac{P_j}{P_i} = \frac{u_{js}^* k_{js} K_{js}^+}{u_{is}^* k_{is} K_{is}^+}. \tag{2}$$

No assumptions as to electroneutrality or as to profiles of potential or concentration were necessary to obtain this result, but it was assumed that the equilibria at the membrane-solution interfaces were not perturbed by the flux of the complexes.

The membrane conductance in the limit of vanishingly small currents was also deduced in paper I for the equilibrium situation where the aqueous solutions on both sides of the membrane have the same composition by evaluating the concentration profiles through integration of the Poisson-

1 For simplicity in referring to equations from papers I and II, we will denote these in the forms "Eq. (1, I)" or Eq. (1, II)" to signify Eq. (1) of paper I or Eq. (1) of paper II, respectively.

Boltzmann equation. For the case of a single salt to be studied here and for the negligible concentration of complexes in the aqueous media expected for the macrotetralides, the conductance is given simply by Eq. (59, I) as:

$$G_0(J)=\left[\frac{F^2}{d}u^*_{js}k_{js}K^+_{js}\right]C^{\mathrm{Tot}}_s a_j. \tag{3}$$

Eq. (3) indicates that the membrane conductance is expected to be proportional to the total concentration of antibiotic in the aqueous solution, C^{Tot}_s, and also proportional to the activity of the cation J^+ in the solution. The proportionality constant, indicated by brackets in Eq. (3), is seen to depend on the parameters $u^*_{js}k_{js}K^+_{js}$ as well as on the membrane thickness. The same combination of parameters also determines the permeability ratio [Eq. (2)]. Thus, one test of the model is to compare measurements of membrane potential and conductance. In particular, considering the ratio of $G_0(J)$ to $G_0(I)$, measured at the same antibiotic and salt concentrations and assuming the membrane thickness to be constant, Eq. (3) gives:

$$\frac{G_0(J)}{G_0(I)}=\frac{u^*_{js}k_{js}K^+_{js}}{u^*_{is}k_{is}K^+_{is}}. \tag{4}$$

Thus, recalling Eq. (2):

$$\frac{G_0(J)}{G_0(I)}=\frac{P_j}{P_i}. \tag{5}$$

The conductance ratio is therefore expected to be identical to the permeability ratio.

The experimental results to be presented here will demonstrate the success with which the theoretical Eqs. (1) and (3) describe the observed properties of phospholipid bilayer membranes in the presence of the macrotetralide antibiotics; the results will also verify the constancy of the parameters P_j/P_i expected from Eq. (2) for a wide range of aqueous antibiotic and salt concentrations, as well as the close agreement between conductance ratios and permeability ratios expected from Eq. (5).

Prediction of Membrane Properties from Salt Extraction Equilibrium Constants

By a detailed analysis of the chemistry of salt extraction equilibrium, it was shown in paper II that the main electrical properties of bilayer membranes observed in the presence of molecules forming "isosteric" complexes (i.e., complexes whose size and shape is the same for all cations bound) can be predicted from measured values of salt extraction equilibrium constants. In particular, it was shown that membrane permeability ratios

and conductance ratios are expected to be equal to the ratio of the equilibrium constants of salt extraction into a convenient solvent [*see* Eq. (15, II)]:

$$\frac{P_j}{P_i}=\frac{K_j}{K_i}; \tag{6}$$

$$\frac{G_0(J)}{G_0(I)}=\frac{K_j}{K_i}. \tag{7}$$

This result permits one to predict, through the use of Eq. (1), the zero current potential behavior of bilayer membranes in the presence of the macrotetralide actins.

The dependence of the individual membrane conductances on the values of K_i can also be obtained by rewriting the expression of the membrane conductance [Eq. (3)] in the form:

$$G_0(I)=A_i K_{is}^{+} \tag{8}$$

where A_i, defined as:

$$A_i=\frac{F^2}{d}u_{is}^{*}k_{is}C_s^{\mathrm{Tot}}a_i, \tag{9}$$

is a characteristic constant of the cation for measurements carried out at a given concentration of antibiotic and salt.

To relate K_{is}^{+} to the salt extraction parameters, Eq. (8, II) can be used in the form

$$K_i=B_i K_{is}^{+} \tag{10}$$

where B_i is a characteristic constant for each cation defined in terms of partition parameters:

$$B_i=\frac{k_{is}k_x}{k_s}. \tag{11}$$

Introducing Eq. (10) into Eq. (8), we obtain

$$G_0(I)=\frac{A_i}{B_i}K_i, \tag{12}$$

predicting a direct proportionality between the membrane conductance and the value of the salt extraction equilibrium constant. Although this equation is valid in general, the proportionality constant A_i/B_i is independent of the cation only if the complexes are "isosteric" (recall from paper II that in this case k_{is} and u_{is}^{*} are the same for all cations). Therefore, if the complexes are "isosteric", a single proportionality constant is expected

to relate membrane conductances to salt extraction equilibrium constants for all cations.

The extent to which the bilayer propertiers are predictable from the salt extraction data of paper II using Eqs. (6), (7) and (12), will be examined in the second part of this paper.

Methods

Salt solutions were prepared using reagent grade chemicals. Rubidium and cesium chlorides, obtained from Penn Rare Metals, were of better than 99.9% purity. Distilled water, deionized with a Barnstead mixed-bed ion exchanger (to a conductivity of less than 0.1 ppm as NaCl) and then redistilled in a Corning Pyrex still, was used to prepare all aqueous solutions, unless noted otherwise. Samples of nonactin, monactin, dinactin, and trinactin (*see* Fig. 1 of paper I for chemical formulae) used in the experiments were generously supplied by Dr. Hans Bickel of CIBA and were used without further purification. Aqueous solutions of the antibiotics were prepared on the day of their use from small volumes of 10^{-3} M stock solutions in ethanol. It was necessary to use ethanol since the solubility of the antibiotics is very low in water; but the largest ethanol content of the aqueous solutions used in the experiments never exceeded 0.1%, a concentration we have found to have no effect on the electrical properties of bilayers (also *see* Mueller & Rudin, 1967; Andreoli, Tiefenberg & Tosteson, 1967).

The phospholipid was extracted from soy bean lecithin (asolectin, Assoc. Concentrates, Inc., New York) by two similar purification methods. In the case of the lipid used in most experiments, designated as type I, 30 gm of asolectin was dissolved in 24 ml of chloroform, and 34 ml of methanol was then added with continuous stirring. The supernatant was decanted from the precipitate and filtered. The precipitate resulting from addition of 200 ml of acetone to the filtrate was washed with acetone, vacuum-dried, and dissolved in n-decane (Eastman, practical grade) to give a stock solution containing about 100 mg of lipid per ml of decane. In the case of the lipid designated as type II, 20 gm of asolectin was dissolved in 40 ml of 1:1 chloroform:methanol (*v/v*) mixture. The supernatant was decanted from the resulting viscous precipitate, filtered, and reprecipitated by the addition of 200 ml of acetone to the filtrate. The resulting precipitate was washed with acetone, vacuum-dried and dissolved in n-decane, as described above. A stock solution of type II lipid was found to retain its membrane-forming ability and its electrical properties without noticeable changes when stored at 1 °C for 4 months. Although these lipids were indistinguishable in their properties in all our measurements, the type of lipid will be identified in the text to avoid any ambiguity. The lipid was diluted with n-decane to form a solution of 15 mg/ml lipid in decane before the start of each experiment.

Since the properties of the membrane were found to be sensitive to the amount of cholesterol present in the lipid, the effects of a second acetone precipitation were tested. No significant changes were found in the bilayer electrical properties as a result of this, indicating that the first acetone precipitation is sufficient to remove most of the cholesterol. The cholesterol added to the lipid in some experiments was purchased from Eastman (reagent grade).

The chamber used for all of the bilayer work to be described here was machined from a single piece of Teflon and fitted with an optically flat Pyrex window following the design of Marcus Goodall (for diagram, *see* Fig. 3 of Eisenman, Ciani & Szabo, 1968). The septum on which the membranes were to be formed was thinned by milling, and then a small hole was drilled in it. The aperture was polished by friction with a smooth cotton string, a procedure which increased membrane stability. This resulted

in a smooth-walled circular opening of 1.4-mm diameter (measured optically). The volume (20 ml) of the two compartments of the chamber were equal. The chamber and all of its parts were boiled in concentrated NaOH solution for about 10 min prior to first being used. This was necessary not only to clean it but also to stabilize its dimensions (the extruded Teflon was found to expand irreversibly after the first boiling) so that this treatment could be repeated whenever an unquestionably clean chamber was needed. However, we normally used a less violent procedure in which the disassembled chamber was first rinsed in distilled water, followed by acetone, and then in chloroform (for at least 10 min) followed by petroleum ether, and then dried in air. When completely dry and equilibrated at room temperature, the chamber was considered ready for a new experiment. The chamber was shock-mounted on a platform consisting of a 0.25-inch steel plate (10×12 inches) on which two 20-lb. lead bricks were placed and which was supported by fresh tennis balls. This system was found to be particularly effective in damping all but very low frequency vibrations.

At the start of each experiment, carried out at 23 ± 1 °C, the clean chamber was filled with an unbuffered aqueous solution of a given composition. A measured volume (usually 2 to 3 μliters) of lipid in n-decane was introduced with the aid of a disposable Pasteur pipette into the front compartment of the chamber on the septum near the opening for the membrane. Membranes were then formed by passing an air bubble from the pipette over the aperture. This method introduces a known amount of lipid at the beginning of each experiment, which is constant thereafter. A new Pasteur pipette is used for each experiment to avoid contamination. It is important to have only a small quantity of lipid in the chamber since the presence of large amounts may decrease appreciably the aqueous concentration of compounds that partition strongly in favor of the lipid phase. Such effects were shown to be negligible for our experimental arrangement for monactin (Szabo, 1969) and should also be so for the other macrotetralides.

Normally, thinning of the membrane starts at the bottom of the aperture, following the usual sequence of interference colors described by Mueller, Rudin, Tien, and Wescott (1962). Within minutes, the whole membrane area becomes black. A small but visible torus of bulk lipid always connects the bilayer to the supporting septum. The membrane conductances were monitored repeatedly during the thinning process and found to be proportional to the area of black membrane whenever sufficiently high concentrations of antibiotic are present. This indicates that in the presence of antibiotic, the conductance of the bilayer is sufficiently large compared to that of the surrounding torus so that the electrical properties of the torus may be neglected. Membranes were continuously watched through the Pyrex window with a stereomicroscope (fitted with an eyepiece micrometer to measure membrane area), using a fiber-optic light source (Radiation Equipment Co., Chicago) to obtain good illumination of the full area of the membrane. Measurements were made only on planar membranes. In case the membrane bowed, it was made planar by the addition of aqueous solution to the appropriate compartment of the chamber. The area of the membrane was taken to be that of the opening in the Teflon septum (0.0158 cm^2), and no attempts were made to correct for the small torus at the edges of the membrane since measurements were made only when the width of the torus was negligible. In the absence of antibiotics, membranes normally become thin spontaneously in distilled water as well as in solutions of concentration as high as 4.0 M of the alkali metal chlorides; in the presence of the macrotetralides, however, this does not always occur spontaneously. In particular, at antibiotic concentrations higher than 10^{-9} M in the absence of added salt, the membrane thins only to a silver appearance; it is necessary to apply 100 mV DC or AC (10 Hz to 100 kHz) voltages transiently to initiate and complete the thinning process. (When the applied voltage is removed, such membranes slowly revert to a silver appearance, starting from the edges, but this reversion is usually sufficiently slow to permit conductance and potential

measurements to be made for the black area.) Large applied voltages in themselves have no residual effect on the electrical properties of bilayers, as tested by applying them to membranes in which thinning is spontaneous. Further details on the thinning of bilayers in the presence of macrotetralides are given elsewhere (Szabo, 1969).

Membrane potentials and conductances were measured using chlorided silver wire or silver plate electrodes of about 1 cm^2 area. However, in preliminary experiments, saturated KCl-calomel salt bridges were used with identical results. All solutions contained known concentrations of chloride to define the potential of the AgCl electrodes. The electrodes were prepared in pairs by electrolysis in HCl solutions. AgCl electrodes were chosen to eliminate liquid junctions and also to minimize the possibility of contamination by antibiotics and salts. Asymmetry potentials (always less than 1 mV) between the pairs of Ag–AgCl electrodes in solutions of the same composition could be measured conveniently in the absence of the membrane.

Membrane potential and conductance were measured with a Keithley model 601 electrometer. In all cases, the input resistance of the electrometer and its connections was measured to be more than 10^{13} ohms in the absence of electrolyte in the chamber.

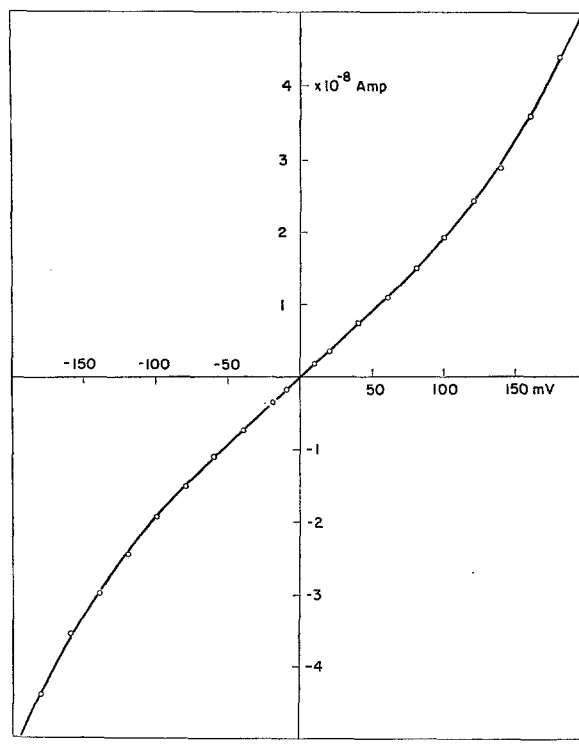

Fig. 1. Current-voltage relationship typical of bilayer membranes in the presence of the macrotetralide antibiotics. The measured values of the steady state membrane current are plotted as a function of the potential difference applied across bilayer membranes formed from type II lipid separating identical solutions having 2.2×10^{-7} M monactin and 10^{-2} M CsCl concentrations. A linear relationship is observed up to ± 60 mV. with slope 1.7×10^{-7} mho. The same linearity was observed for smaller applied voltages (i.e., between 1 and 10 mV.). In the absence of a membrane, the current-voltage relation is linear and has a much larger slope (1×10^{-4} mho), indicating that the two-electrode system used for this experiment introduces negligible errors

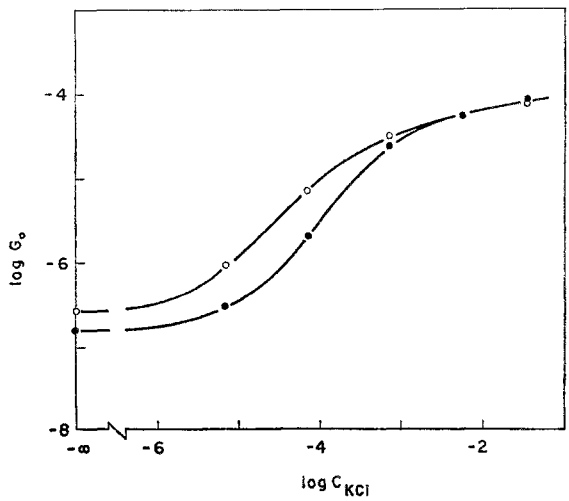

Fig. 2. The effect of stirring on the membrane conductance. Membrane conductances in 10^{-7} M nonactin solution are plotted as a function of increasing KCl concentration in the aqueous phases. Filled circles indicate measurements obtained when the solution was only slightly stirred after each increase in KCl concentration. Open circles represent values measured when stirring was sufficiently strong so that further stirring produced no subsequent effects on the membrane conductance. The conductances in this figure, and throughout the remainder of this paper, are expressed per square centimeter of membrane area and have the units of mho cm^{-2}

This was safely larger than the maximum membrane resistance studied of 10^{11} ohms. Values of the membrane conductance were calculated from the slope of the steady state current-voltage relationship at zero current, a typical example of which is given in Fig. 1. Usually, measurements were made with a two-electrode system by applying DC potential differences to the chlorided silver electrodes and measuring the steady state current. Small potential differences (between +10 and −10 mV) were used so that the resistance was measured within the linear region of the I−V curve. To verify that electrode polarization, electrode resistance, and electrolyte resistance were negligible, control measurements were carried out, as routine, in the absence of a membrane. For membranes of very high conductance, controls were also carried out with a four-electrode voltage clamp. (The circuit, designed and built by R. Wyatt, is given in Fig. 8 of Walker, Eisenman & Sandblom, 1969.)

Strong stirring of the aqueous solutions increases the value of the membrane conductance, as illustrated in Fig. 2 where measurements are compared for gentle vs. strong stirring. However, on vigorous stirring, a limiting value is always reached where further increase in stirring has no effect. Only these limiting values are presented here.

Results

Membrane Properties in the Absence of Antibiotic

In the absence of antibiotic, membranes formed from type II phospholipid were found to be somewhat more permeable to cations than to anions (potential differences of about 30 mV being observed for KCl

when there is a 10-fold difference in KCl concentration across the membrane), in agreement with previous observations of others for similar lipid membranes (Laüger, Lesslauer, Marti & Richter, 1967; Andreoli, Bangham & Tosteson, 1967). A slight selectivity among cations was also observed (e.g., the bi-ionic potential between 0.1 M NaCl and 0.1 M KCl solutions was about 15 mV, KCl side negative), confirming data of Lev, Gotlib, and Buzhinsky (1966). The membrane conductance was typically about 2×10^{-9} mho cm^{-2} and was largely independent of salt concentration in agreement with the previous results of Hanai, Haydon, and Taylor (1965*a*), Lev et al., (1966), and Laüger et al. (1967); whereas the membrane capacitance was 0.4 $\mu f/cm^2$, in good agreement with the results of Hanai et al. (1965*a*).

Membrane Properties in the Presence of Antibiotic

The macrotetralide actin antibiotics markedly increased the membrane conductance for both type I and type II lipids when present even at low concentrations in the aqueous phase. The membrane became selectively permeable to cations (as judged by membrane potentials of 58.5 mV per 10-fold ratio of activity on the two sides of the membrane) with marked selectivity differences among monovalent species, in accord with the previous findings of Mueller et al. (1967), Tosteson (1968), and Eisenman et al. (1968). The presence of a macrotetralide had little effect on the membrane capacitance although precise measurements of this parameter have not been made.

Membrane Potential

The time course of the membrane potential observed in response to step changes in the solution concentration of cations is illustrated in Fig. 3. The step response seen is typical of bilayers in the presence of macrotetralide actins. The lower portion of Fig. 3, obtained in $K^+ - Rb^+$ mixtures, illustrates that the step response is typically observed in mixtures of two cations as well. Following the period of rapid initial change, membrane potentials were found to be time independent for more than 30 min, provided that the aqueous phases were occasionally stirred.

From steady state values such as these, the effects of the macrotetralide antibiotics and cations on the membrane potential were characterized. Fig. 4 summarizes the results obtained in the presence of 10^{-7} M nonactin, monactin, dinactin, or trinactin for mixtures of K^+ with the indicated group Ia cations. The observed potentials are plotted as points as a function of the logarithm of the added KNO_3 concentration; the solid curves demonstrate the potential behavior expected theoretically from Eq. (1) for the

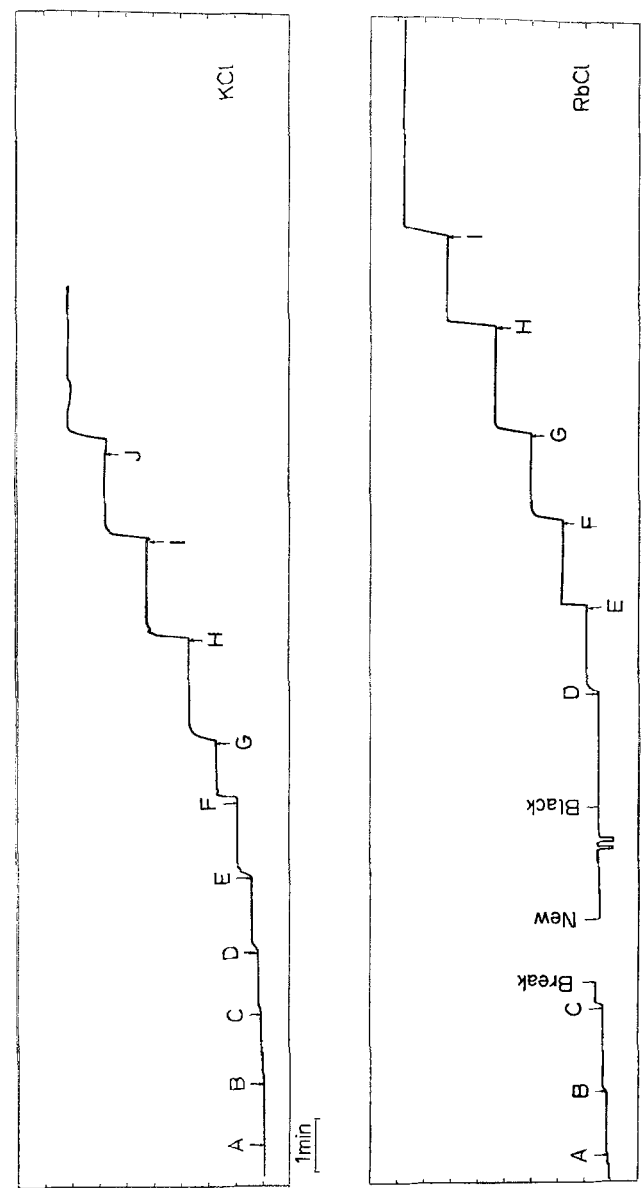

Fig. 3. Typical time course of bilayer membrane potentials following step changes in the solution conditions. Membranes were initially formed from type II lipids in the presence of solutions containing 10^{-7} M monactin in 10^{-3} M KCl (upper) or 10^{-3} M RbCl (lower). At the times marked by arrows, small volumes of KNO_3 were added successively to the front compartment of the chamber to increase the potassium concentration. The solutions were then stirred and the membrane potential was observed to reach its steady value as soon as a uniform composition was obtained. The K^+ concentration (M) in both figures was increased by: $A=1.3\times10^{-5}$; $B=4.6\times10^{-5}$; $C=1.1\times10^{-4}$; $D=2.5\times10^{-4}$; $E=5.6\times10^{-4}$; $F=1.2\times10^{-3}$; $G=2.5\times10^{-3}$; $H=5.7\times10^{-3}$; $I=1.2\times10^{-2}$; $J=2.4\times10^{-2}$. In the lower figure, the membrane broke shortly after addition C. It was formed again without changing the solution and observed to have obtained blackness where indicated. Note that it showed the same membrane potential at this time as before breaking

values of the permeability rations given in the figure. For each of the macrotetralides and all cations, the agreement between the experimental points and the theoretical curves is seen to be excellent.

Table 1. *The independence of the P_i/P_K ratios of the aqueous concentration of the macrotetralides* [a]

Ratio	$C_{Monactin}$	
	5×10^{-11} M	10^{-7} M
P_{Cs}/P_K	0.029	0.023
P_{Rb}/P_K	0.37	0.47

[a] The membranes were formed in 10^{-2} M chloride solutions. The data at 5×10^{-11} M were obtained with membranes of Type II lipid. The data at 10^{-7} M were obtained with membranes of Type I lipid.

The permeability ratios are independent of the concentration of the macrotetralide. This is illustrated by the data of Table 1 where a 2,000-fold change in the monactin concentration is seen to have little effect on the values of the permeability ratios tabulated there. The permeability ratio is also independent of the salt concentration. This can be seen by the agreement, over the wide range of variation of the KNO_3 concentration in Fig. 4, of the experimental points with the theoretical curves drawn to constant permeability ratios. Further evidence for such independence is seen in the excellent agreement between the P_{Rb}/P_K values of Fig. 4 and those of Fig. 3 obtained for 10-times-more-dilute solutions ($P_{Rb}/P_K = 0.5$ for Fig. 3 and 0.47 for Fig. 4).

We therefore conclude that the potential behavior in the presence of the macrotetralide actins is described satisfactorily by Eq. (1) over a wide range of experimental conditions.

Comparing the values of permeability ratios of Fig. 4 among the macrotetralides, the sequence of relative permeabilities is the same for all antibiotics – K > Rb > Cs > Na > Li – although quantitative differences are observable from one antibiotic to another, which are consistent with those discussed in paper II.

Membrane Conductance

The dependence of membrane conductance (measured at zero current with the same solutions on both sides) on the aqueous concentration of antibiotic is illustrated in Fig. 5 for monactin in the presence of 10^{-2} M (left) and 10^{-1} M (right) concentrations of the indicated alkali metal cations. The experimental points can be seen to agree with the lines of unit slope expected from Eq. (3) over a range of many orders of magnitude of monactin

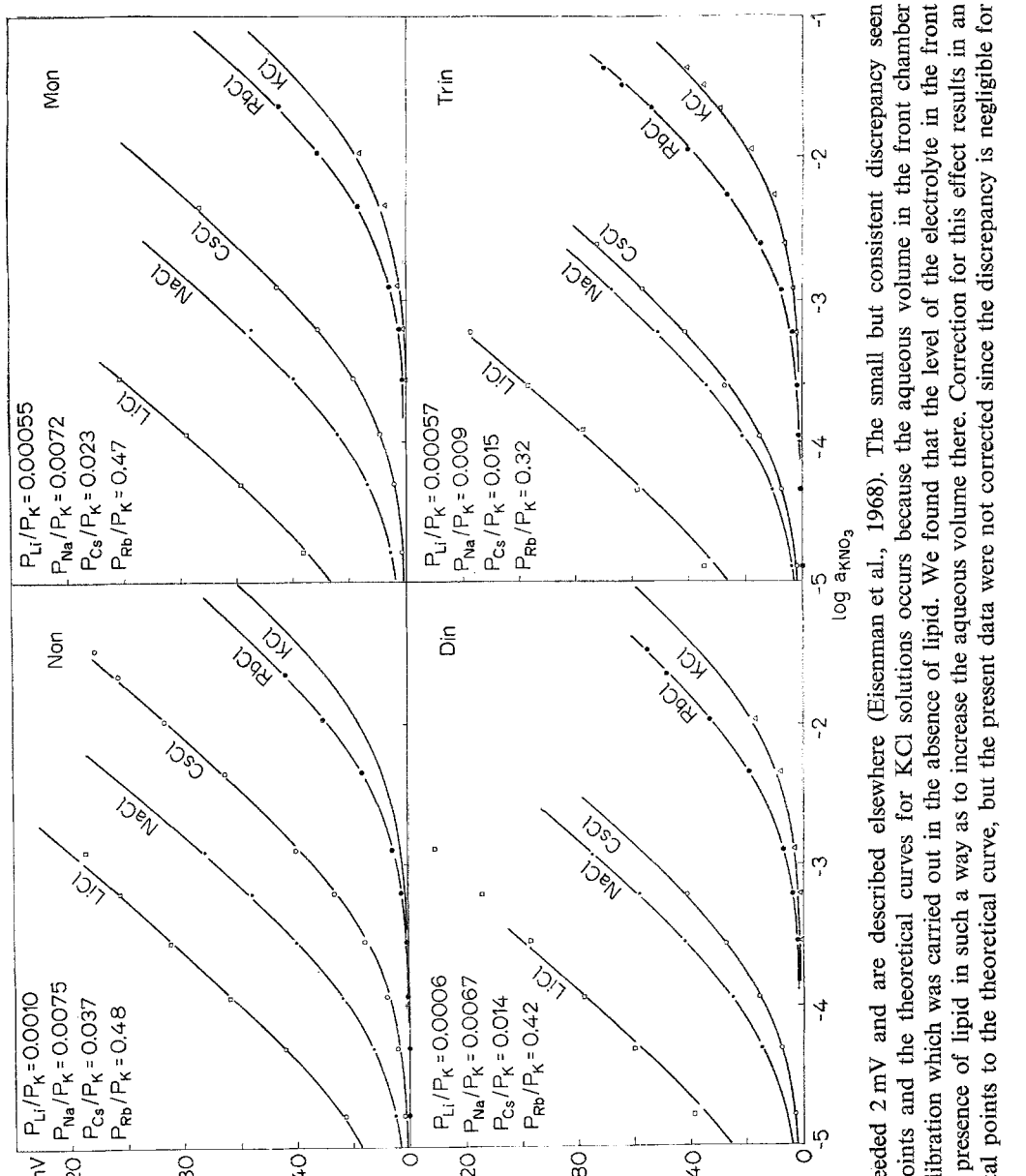

Fig. 4. Membrane potentials in alkali metal chloride-potassium nitrate mixtures in the presence of nonactin, monactin, dinactin, or trinactin. Membranes were formed from type I lipid in 10^{-2} M alkali metal chloride solution at 10^{-7} M concentration of the indicated macrotetralide actin (non-, mon, din-, trin-). The plotted values of the steady state potentials were observed in the manner of Fig. 3. For comparison, the curves are drawn according to the theoretical expectations of Eq. (1) for the indicated values of the permeability ratios. For additions of KNO_3 larger than 10^{-2} M, small corrections of the observed potential were made to take into account the effects of ionic strength on the activity coefficient of Cl^- (recall that AgCl electrodes are being used).

These corrections never exceeded 2 mV and are described elsewhere (Eisenman et al., 1968). The small but consistent discrepancy seen between the experimental points and the theoretical curves for KCl solutions occurs because the aqueous volume in the front chamber is underestimated by the calibration which was carried out in the absence of lipid. We found that the level of the electrolyte in the front chamber was altered by the presence of lipid in such a way as to increase the aqueous volume there. Correction for this effect results in an agreement of the experimental points to the theoretical curve, but the present data were not corrected since the discrepancy is negligible for

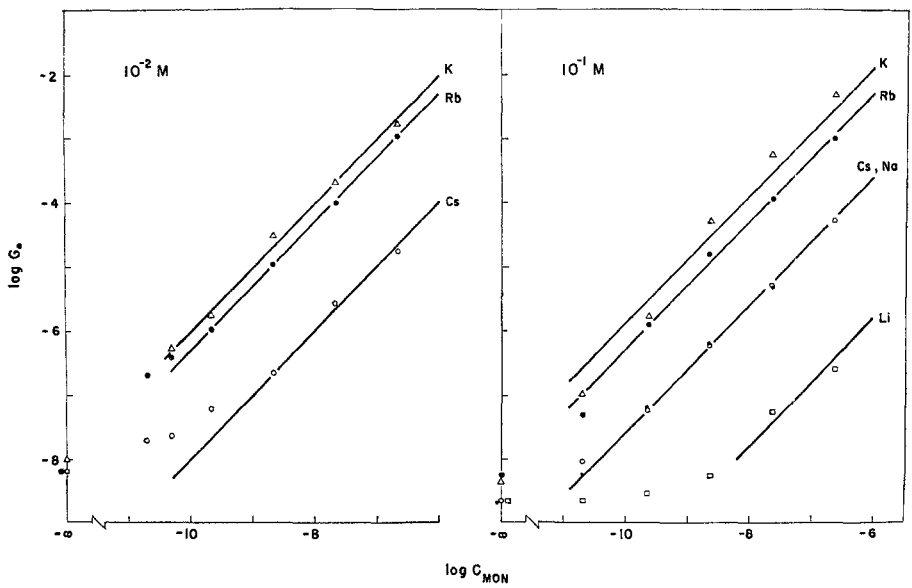

Fig. 5. The proportionality between membrane conductance and the concentration of monactin. Type II lipid. Abscissa: Logarithm of the aqueous monactin concentration. Ordinate: Logarithm of the membrane conductance in 10^{-2} M (left) and 10^{-1} M (right) alkali metal chlorides. Points indicate the experimentally observed values; the solid lines were drawn to unit slope expected for the proportionality between the membrane conductance and monactin concentration deduced from Eq. (3). The monactin concentration was increased by successive additions of small volumes of monactin stock solution to both compartments of the chamber. Pyrex-redistilled distilled water (not deionized) was used in these experiments

concentration; but, of course, the membrane conductance at low antibiotic concentrations cannot fall below the finite value due to the residual conductance of the membrane in the absence of monactin (the data points at the extreme left of the figure). The results are typical of the other macrotetralides as well and are similar to previous observations by Tosteson (1968) for monactin-dinactin mixtures. They indicate that the membrane conductance is indeed directly proportional to the aqueous concentration of the macrotetralide antibiotic, as expected theoretically from Eq. (3). This proportionality exists over a very wide range of salt concentrations, as demonstrated in Fig. 6. Here, a 100-fold increase of nonactin concentration from 10^{-9} to 10^{-7} M is seen to result in a 100-fold increase in membrane conductance over the entire range of KCl concentration from less than 10^{-5} M to greater than 0.1 M.

The dependence of membrane conductance on aqueous salt concentration is illustrated in Figs. 7–10. For a constant level of antibiotic

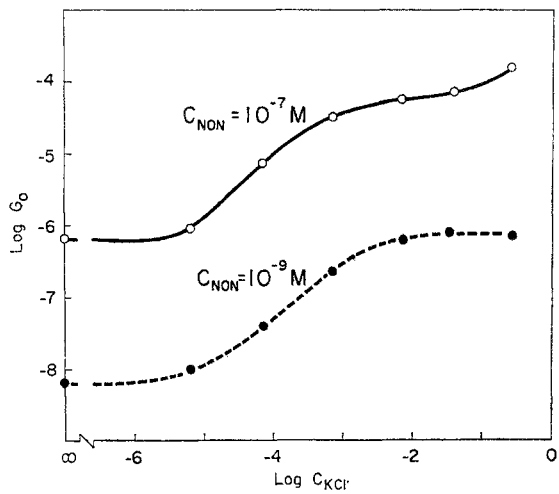

Fig. 6. The proportionality between the membrane conductance and the aqueous monactin concentration seen over a range of ionic strengths. Type I lipid. The logarithm of the membrane conductance in the presence of 10^{-9} and 10^{-7} M monactin is plotted as a function of the logarithm of the aqueous KCl concentration. Note the 100-fold difference in conductance observed for a 100-fold difference in nonactin concentration of each of the KCl concentrations. The concentration of KCl was increased by adding small volumes to both compartments of the chamber. The large membrane conductance observed at the left in the presence of 10^{-7} M nonactin, but in the absence of any added KCl, is due to the presence of trace ionic contaminants (most likely NH_4^+) in the distilled water

concentration, the conductance is expected from Eq. (3) to be directly proportional to the activity of the cation in the aqueous solution; such a proportionality is seen in the presence of nonactin, monactin, and trinactin from the data of Fig. 7, although only over a limited concentration range. Deviations from this proportionality occur at salt concentrations higher than 10^{-3} M. For monactin, this deviation takes the form of an apparently simple "saturation", whereas for nonactin and trinactin, the behavior is more complex. These deviations will be shown to be understandable, and indeed expected, in terms of the present theory once we recognize that such physical properties of the lipid bilayer as its "viscosity" are expected to vary with ionic strength (Van Deenen, 1969). To clarify this point, however, we must first characterize the experimental phenomena in considerably greater detail, as we will now do.

Complex dependence of the membrane conductance on salt concentration is characteristic of all the alkali metal cations. This is illustrated in

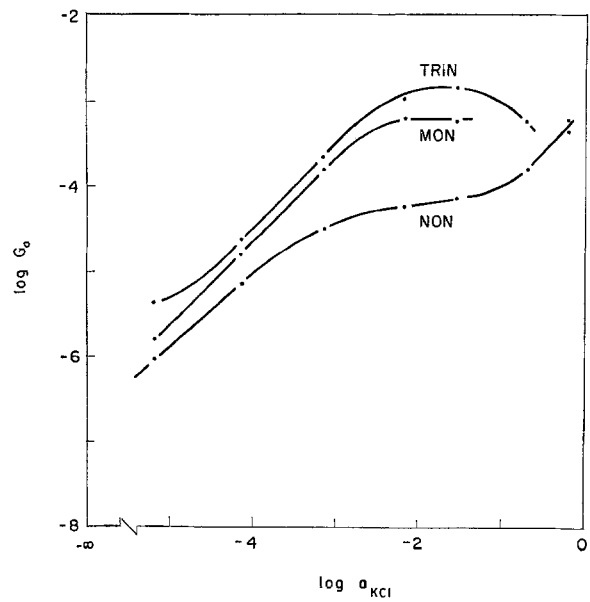

Fig. 7. The dependence of membrane conductance on the aqueous KCl activity. Type I lipid. Abscissa: Logarithm of the aqueous potassium chloride activity. Ordinate: Logarithm of the membrane conductances observed in the presence of 10^{-7} M nonactin, monactin, and trinactin. The curves are not theoretical but merely connect experimental points. The decrease of membrane conductance for trinactin at high KCl concentration is likely to be due to the formation of significant amounts of K^+-complex in the aqueous phases (*see* Eq. 60, I) together with the effects of ionic strength to be discussed (*see* Fig. 9)

Fig. 8 for nonactin, where, in the case of LiCl, the membrane conductance even *decreases* with increasing concentration. However, despite the superficial differences from cation to cation (e.g., the apparent "saturation" in the case of NH_4^+, K^+, Rb^+, and Cs^+, the apparent concentration-independence in the case of Na^+, and the inverse concentration-dependence in the case of Li^+), all of these observations are what would be expected if, in addition to the simple increase of conductance with increasing cation activity expected from Eq. (3) for a lipid of constant "viscosity", the increasing ionic strength were to alter the physical properties of the lipid (e.g., its "viscosity") and hence decrease the mobilities of all complexes within its interior.

That this is indeed the case is seen by carrying out a simple experiment in which one studies the effects of increasing the salt concentration of a poorly permeating species (e.g., Li^+) on the conductance of a highly permeant species (e.g., K^+). Such an experiment is illustrated in Fig. 9 at the

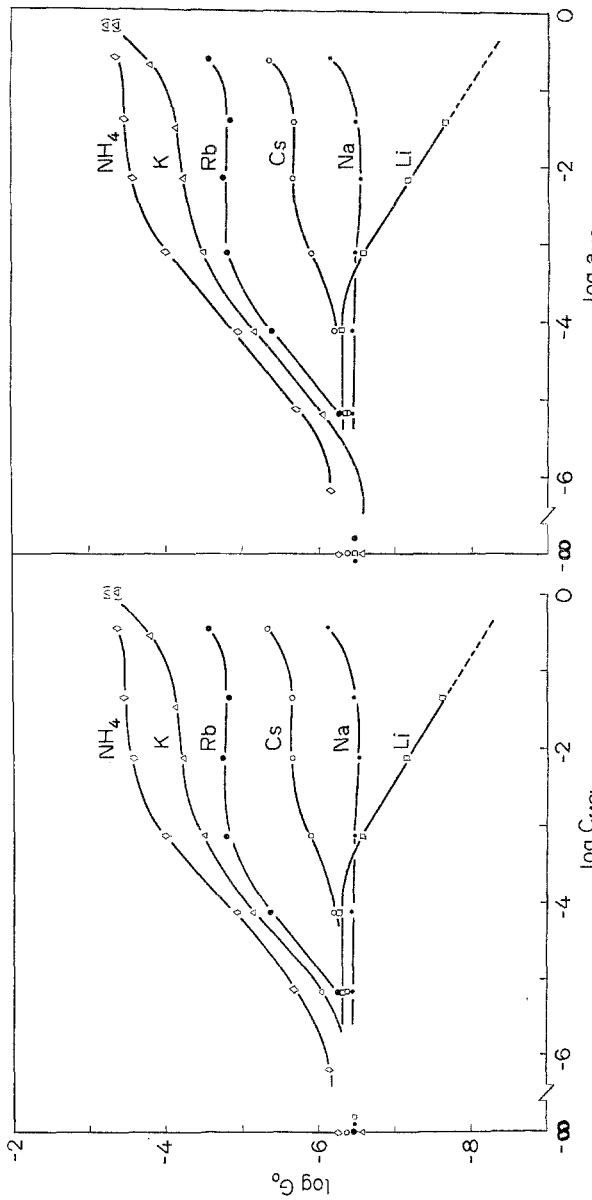

Fig. 8. The dependence of membrane conductance on aqueous salt concentration and activities. Type I lipid. 10^{-7} M nonactin. Left: The logarithm of membrane conductances is plotted against the aqueous chloride concentration of the indicated cations. Right: These data are plotted as a function of aqueous activities. The curves are not theoretical but merely connect the experimental points. The KCl data are the same as those of Fig. 7. Bracketed points for KCl were obtained in separate experiments

left, where the effects of adding LiCl to solutions initially containing KCl at three different concentrations (10^{-5}, 10^{-4}, and 10^{-3} M) are illustrated. Since Li^+ itself should carry very little current across the membrane (*see* Fig. 8) in the presence of nonactin, its effects in Fig. 9 should be referable primarily to its effects on the K^+ conductance of the membrane. For the present theory, if the membrane's physical properties were unaltered by

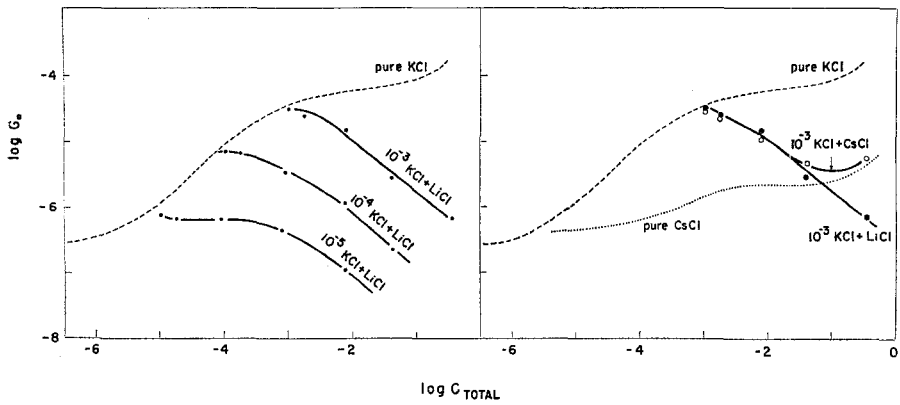

Fig. 9. Effects of ionic strength on the K^+ conductance of bilayers. Type I lipid. 10^{-7} M nonactin. The effect on the membrane conductance in 10^{-3}, 10^{-4}, or 10^{-5} M KCl, of increasing LiCl concentration is shown on the left where the logarithm of the membrane conductance is plotted as a function of the logarithm of the total salt concentration. The curve labelled "pure KCl" is traced from Fig. 8. LiCl is seen to decrease markedly the conductance due to K^+. The similar plot at the right compares the effect of increasing the total salt concentrations with CsCl (open circles) against that of LiCl (filled circles) in the presence of 10^{-3} M KCl. Notice the identical effects of LiCl and CsCl at those CsCl concentrations for which the conductance due to Cs is negligible (*see* curve labelled "pure CsCl", which is traced from Fig. 8)

adding LiCl, there should be no change in the total measured conductance (i.e., level curves should be obtained) since the Li^+ conductance is negligible. Indeed, if Li^+ were to contribute to the conductance, the latter should increase. In contrast to these expectations, the conductance is seen to *decrease* with increasing LiCl concentration, the effect being first seen at about the same ionic strength (i.e., 10^{-4} M) for each of the three K^+ concentrations tested.[2]

This behavior of the membrane conductance is due to the ionic strength increase produced by adding LiCl, rather than being an effect specific to the Li^+ ion, since (as shown at the right of Fig. 9) identical results are obtained when CsCl is added instead of LiCl. Here it is clear that the effects of Cs^+ (open circles) and Li^+ (filled circles) are indistinguishable up to those concentrations at which the conductance of Cs^+ itself becomes

2 The effect of increasing the ionic strength with Li^+ is not on the antibiotic molecules by themselves. This was shown in paper II where the extraction of potassium picrate into dichloromethane, induced by the presence of monactin, was observed to be unaltered when the ionic strength was increased by adding Li^+ salt at concentrations comparable to those of the bilayer experiments. A similar conclusion holds for Ca^{2+}, Mg^{2+} and Th^{4+} ions whose presence has no effect on salt extraction but which reduce strongly the conductance of bilayer membranes, as will be seen shortly.

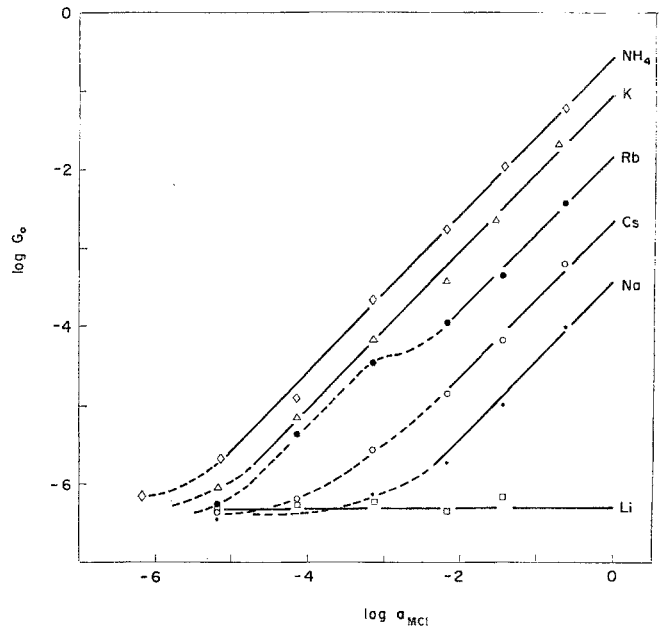

Fig. 10. Membrane conductances corrected for the postulated effect of ionic strength on the physical properties of the lipid. The observed effect of LiCl on the K^+ conductance of bilayers in 10^{-4} M KCl solution from Fig. 9 is used to correct the data of Fig. 8 for such an effect on all cations. This is done by multiplying the conductances on the right of Fig. 8 by a factor equal to the decrease observed on the curve of Fig. 9 labelled "10^{-4} KCl + LiCl" at the same total ionic strength

important. Therefore, the effects of these species are caused by the increase in ionic strength, not by a specific effect of the ion. This is further supported by the fact that the pure K^+ curve (dashed) is seen to deviate from unit slope at about this ionic strength as well. Similar deviations are present for the other cations in the data of Fig. 8, as will be understood from the following argument.

If the complexities of the data of Fig. 8 were due to an effect of ionic strength alone, this effect should be seen equally on each of the curves, and we ought to be able to correct these data for the ionic strength effect using the results of Fig. 9. We have done this in Fig. 10, where the data of Fig. 8 are corrected using the separately observed effects of LiCl (Fig. 9). The success of this correction is seen in how well the data points fall on the solid lines of a unit slope.

A more direct test of the postulate that the complexities in the conductance vs. concentration curve are due to ionic strength is given by Fig. 11 where the membrane conductance has been studied under conditions in

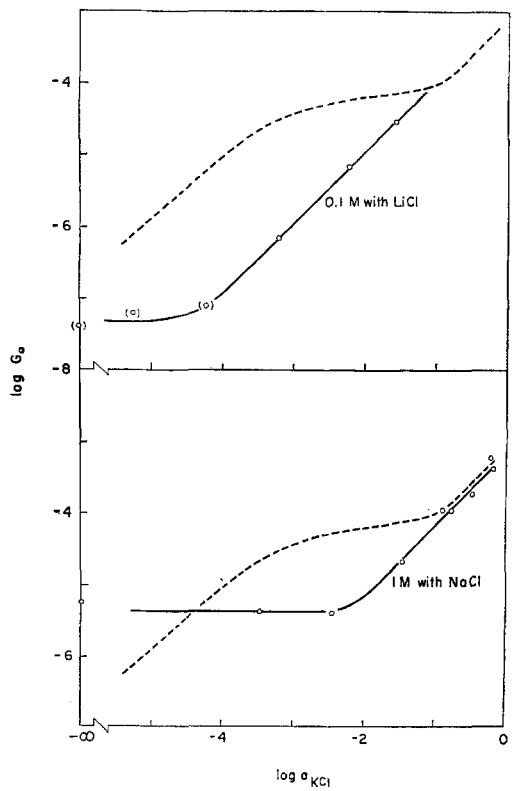

Fig. 11. The proportionality between membrane conductance and K^+ activity when ionic strength is held constant. Type I lipid, 10^{-7} M nonactin. The upper figure plots the conductance of a membrane formed in 0.1 M LiCl as a function of increasing the KCl concentration by addition of small volumes of stock solutions. These additions did not alter appreciably the total ionic strength. Note that the experimental points fall on the solid line of unit slope which intersects the broken line for pure KCl exactly where expected. Parenthesized points indicate imperfectly thinned membranes. The lower figure presents a similar experiment where the ionic strength was maintained at 1 M with NaCl. The four points at the lowest concentrations of KCl were obtained by adding KCl to 1.0 M NaCl. For these points, ionic strength was effectively constant. The points at higher KCl concentrations were measured in separate experiments in mixtures in which NaCl was replaced by KCl so as to hold the ionic strength constant at 1 M. Notice that at low KCl activities, a constant membrane conductance is observed since the Na^+ conductance of the bilayer is not negligible there. The dushed curves are traced from Fig. 8

which the ionic strength is held constant using Li^+ (upper) or Na^+ (lower). Under these conditions, the theoretically expected proportionality between membrane conductance and K^+ activity is clearly seen, as shown by the satisfactory extent to which the experimental points fall on the lines of unit slope at the higher KCl activities where the residual conductance of the bilayer (due to the Li^+ or Na^+ ions) is negligible.

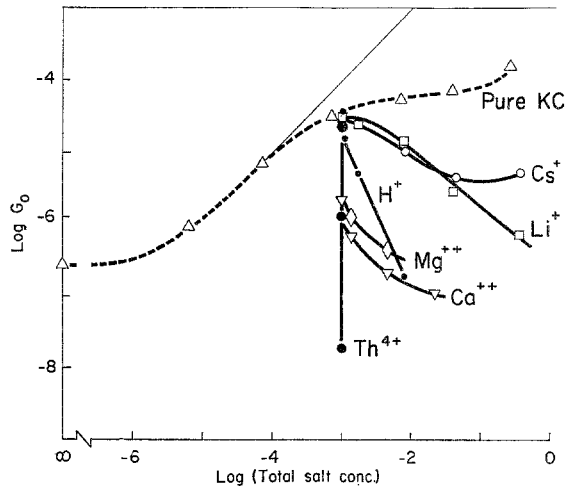

Fig. 12. The effect of H^+, Ca^{2+}, Mg^{2+}, and Th^{4+} on the K^+ conductance of bilayer membranes. Type I lipid. 10^{-7} M nonactin. 10^{-3} M KCl. The dashed curve is for pure KCl as in Fig. 8. The solid curves represent the effects of adding the indicated species in the following amounts to increase the total salt concentration as shown (H^+: 6.7×10^{-6}, 6.7×10^{-5}, 6.7×10^{-4}, 6.7×10^{-3} M; Mg^{2+}: 3.4×10^{-4}, 3.4×10^{-3} M; Ca^{2+}: 3.4×10^{-5}, 3.4×10^{-4}, 3.4×10^{-3}, 1.7×10^{-2} M; Th^{4+}: 0, 6.7×10^{-7}, 6.7×10^{-6} M)

We must therefore conclude that the apparently complex behavior of the membrane conductance in salt solutions of varied concentration is, at least in part, due to an effect of varying ionic strength in itself, and is independent of which alkali metal cation specie is present. When the ionic strength is held constant, the direct proportionality expected from Eq. (3) between membrane conductance and aqueous cation activity is indeed observed. It should come as no surprise that experiments, performed at variable ionic strength in a membrane of labile structure, might be complex to interpret.[3]

It should be further emphasized that a pure effect of ionic strength is observed only for the alkali metal cations. Indeed, Fig. 12 illustrates that H^+, Ca^{2+}, Mg^{2+} and Th^{4+} all have more complex specific effects on the K^+ conductance of bilayer membranes. These effects are likely to involve specific interaction of these ions with the polar head groups of the phospholipids.

3 With these results in hand, it appears that the pure ionic strength effect is most directly illustrated in Fig. 7 by the curve for nonactin. The downturn for trinactin would, in the present theoretical framework, be a consequence of the formation of a significant number of KS^+ complexes in the aqueous phase [through the effects of this in Eq. (60, I)], whereas the nearly horizontal curve for monactin would represent a smaller degree of formation of such aqueous complexes.

The Equality between Conductance Ratios and Permeability Ratios

One of the principle expectations of the theoretical treatment of paper I is the identity [*see* Eq. (5)] of permeability ratios obtained from the membrane potential studies and the ratio of membrane conductances measured at the same concentration of antibiotic and salt for the various cations. We have seen above that the permeability ratio is a constant over a wide range of antibiotic and salt concentrations. Such constancy is also true for the ratio of conductances measured at comparable antibiotic and salt concentrations, as indicated by the corrected data of Fig. 10 (or the raw data of Fig. 8), as well as by the constant displacements between the unit slope lines for the various cations of Fig. 5. The same sequence of membrane permeabilities and conductances was observed in these data.

In order to test the equality of ratios more precisely, we carefully compared the conductances of bilayer membranes among the alkali cations under the same conditions of antibiotic and salt concentration (i.e., 10^{-7} M antibiotic and 10^{-2} M alkali chloride) as previously used in the membrane potential measurements (Fig. 4). The results of these measurements are summarized in Table 2, and the ratios of these conductances are compared with the corresponding permeability ratios in Table 3. The agreement between permeability and conductance ratios is seen to be verified only with the exception of the parenthesized trinactin data for Li^+ and Na^+ for which the membrane did not thin completely. Even in these cases, the agreement is still qualitatively satisfactory.[4] The trends among the macrotetralide actins are best seen by presenting these results graphically, as in Fig. 13 where the permeability ratios (solid lines) are compared to the

Table 2. *Conductances of bilayer membranes in 10^{-2} M alkali metal chloride, 10^{-7} M macrotetralide solutions* [a]

Ion	Macrotetralide			
	Nonactin	Monactin	Dinactin	Trinactin
Li	3.7×10^{-8}	1.1×10^{-7}	2.3×10^{-7}	(5.1×10^{-8})
Na	5.9×10^{-7}	2.1×10^{-6}	9.0×10^{-6}	(6.7×10^{-6})
K	9.0×10^{-5}	4.4×10^{-4}	1.1×10^{-3}	1.5×10^{-3}
Rb	4.2×10^{-5}	1.5×10^{-4}	5.5×10^{-4}	5.8×10^{-4}
Cs	3.4×10^{-6}	6.2×10^{-6}	1.5×10^{-5}	2.0×10^{-5}

[a] Units are in mho cm^{-2}. Parenthesized values were obtained on imperfectly thinned membranes.

4 The ratios in Table 3 have been expressed relative to Rb^+ rather than K^+ because larger discrepancies between the two sets of measurements occur in the case of K^+.

Table 3. *Comparison of permeability and conductance ratios of bilayers observed in the presence of macrotetralide actins* [a]

Ion	Macrotetralide							
	Nonactin		Monactin		Dinactin		Trinactin	
	G_i/G_{Rb}	P_i/P_{Rb}	G_i/G_{Rb}	P_i/P_{Rb}	G_i/G_{Rb}	P_i/P_{Rb}	G_i/G_{Rb}	P_i/P_{Rb}
Li	0.00088	0.0021	0.00073	0.0011	0.00042	0.0014	(0.000087)	(0.0018)
Na	0.014	0.015	0.014	0.015	0.0165	0.016	(0.011)	(0.028)
Cs	0.082	0.077	0.042	0.047	0.027	0.033	0.034	0.047
Rb	1	1	1	1	1	1	1	1
K	2.1	2.1	2.9	2.0	2.1	2.4	2.6	3.1

[a] Parenthesized values were obtained from imperfectly thinned membranes.

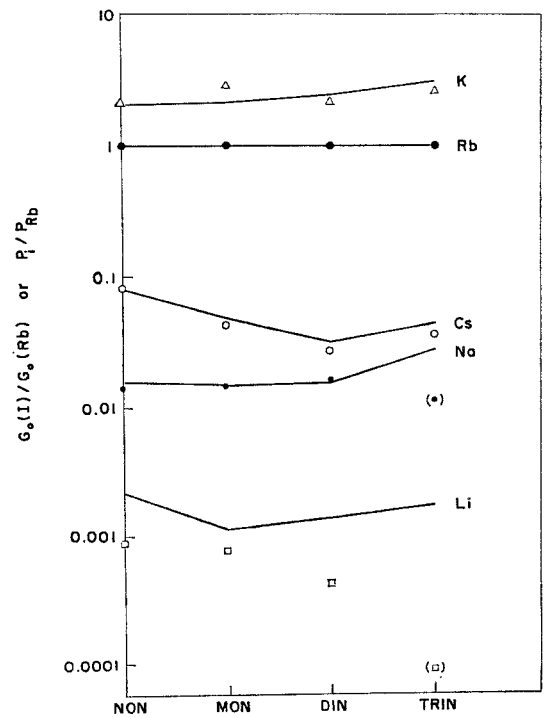

Fig. 13. The close correspondence between conductance ratios and permeability ratios. Type I lipid. 10^{-7} M macrotetralide. The permeability ratios are indicated by solid lines; the conductance ratios are plotted as points for each of the macrotetralide actins which are arranged on the abscissa in sequence of increasing number of methyl side groups. Data originate from Table 3 where the parentheses are defined

conductance ratios (points). Not only are the permeability ratios and conductance ratios among the various cations seen to agree quantitatively (except for Li) for each antibiotic, but also the same systematic changes in these ratios are observed as one proceeds from nonactin to trinactin.

The most notable changes are the decrease of the Cs permeability (or conductance) relative to Rb, and the tendency of the Na permeability (or conductance) to approach that of Cs.

The Effect of Cholesterol on the Electrical Properties of the Membrane

A primary consequence of the postulated role of the macrotetralide actins as a "carrier" for cations is the necessity that the complex be able to move within the membrane. This appears explicitly in the expected dependence of the membrane conductance on the mobility of the complex, as seen in Eq. (3). The mobility should depend not only on the size of the complex but also on the physical properties of the bilayer (e.g., the "viscosity" of the interior through which the complex must move). Indeed, if the interior of the membrane were not liquid-like, the carrier mechanism which we have postulated would be highly unlikely.

We therefore added cholesterol to the lipid from which the bilayers were formed in an attempt to decrease the mobility of the complexes. Cholesterol was chosen because it is known to decrease the permeability to neutral molecules of liposomes (De Gier, Mandersloot & Van Deenen, 1968), and of bilayers (Finkelstein & Cass, 1967, 1968; Bean, Shepherd & Cahn, 1968). Also, its action is thought to be a consequence of an immobilization of the hydrocarbon tails of the phospholipids in the bilayer (Rand &

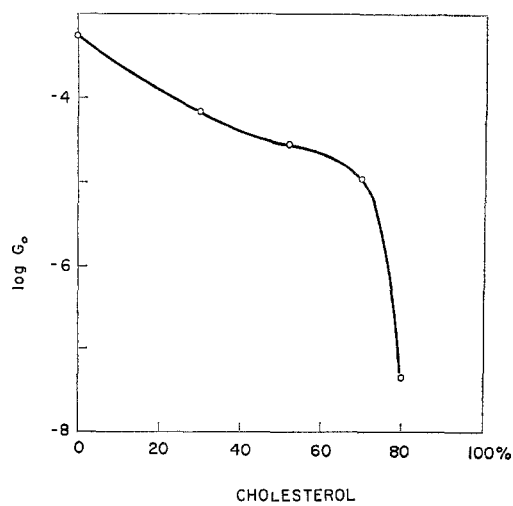

Fig. 14. The effect of cholesterol on membrane conductance. 10^{-7} M monactin. 7.4×10^{-3} M KCl. Abscissa: Weight percent of cholesterol in the type I lipid used to form the membrane. Ordinate: Logarithm of membrane conductance. It was observed that above 70%, cholesterol tends to precipitate from the lipid mixture. For further details, *see* Figs. 15 and 16. The exact lipid compositions used are those shown on Fig. 16; the lipid at 69% cholesterol contained 9 mg of type I phospholipid and 20 mg of cholesterol per ml of n-decane

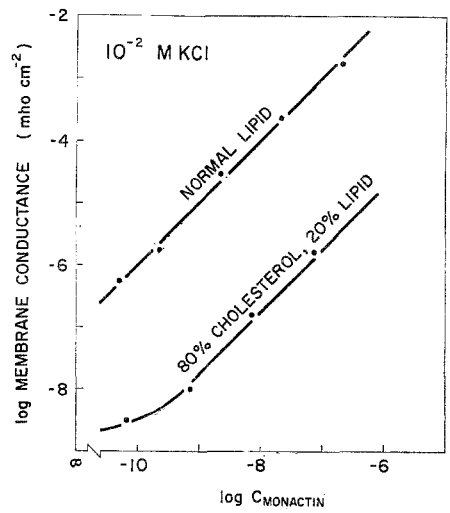

Fig. 15. Proportionality between membrane conductance and aqueous monactin concentration in the presence (and absence) of cholesterol. 10^{-2} M KCl solutions. The experimental points fall on straight lines of unit slope, in the presence of cholesterol as well as in its absence. The data labelled "normal lipid" are from the right of Fig. 5. Lipid composition (labelled "80% cholesterol–20% lipid"): 4 mg type I lipid, 16 mg cholesterol per ml of decane

Luzzalli, 1968; Chapman & Penkett, 1966), in accord with its condensing effect on monolayers (Van Deenen, Houtsmiller, de Haas & Mulder, 1962). Although the effect of cholesterol in solvent-containing membranes like ours might, alternatively, result from a displacement of the solvent (decane), its presence should also impede the movement of the complex.

It seems likely, therefore, that adding cholesterol should decrease the membrane conductance if a carrier mechanism is involved; although if the macrotetralide molecules act by forming pores (as suggested by Mueller et al., 1967), no explicit prediction can be made.

The effect of cholesterol to decrease membrane conductance is illustrated in Fig. 14, where the logarithm of the membrane conductance is plotted as a function of the weight percent of cholesterol in the lipid from which the membrane was formed. (The amount of cholesterol in the bilayer is not known but is likely to vary directly with the amount in the bulk lipid.) The membrane conductance is seen to decrease by nearly 100-fold at about 70% cholesterol content of the lipid. At higher cholesterol contents, cholesterol precipitates from the decane-phospholipid mixture with a large concomitant decrease of membrane conductance. It seems that in effect we have "frozen" the membrane at this point, and, by thus making it

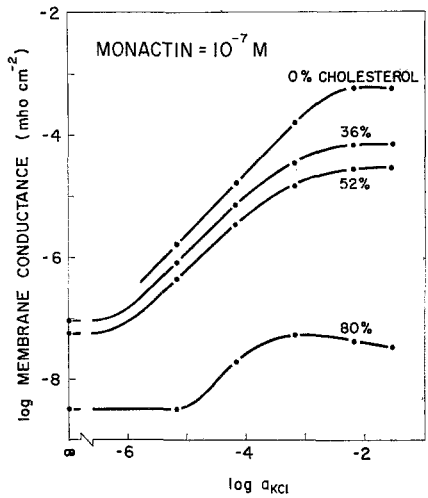

Fig. 16. The effect of cholesterol on the membrane conductance due to nonactin as a function of the aqueous KCl concentration. 10^{-7} M monactin. The composition of the lipids were as follows: 0% cholesterol, 15 mg type I lipid per ml decane; 36% cholesterol, 15 mg type I lipid and 8.3 mg cholesterol per ml decane; 52% cholesterol, 15 mg type I lipid and 16 mg cholesterol per ml decane; 80% cholesterol, 4 mg type I lipid and 16 mg cholesterol per ml decane. Comparing the 80% cholesterol curves between Figs. 15 and 16, note that a discrepancy is seen between the conductances for 10^{-2} M KCl and 10^{-7} M monactin in these two figures, the conductance on Fig. 15 being larger than that of Fig. 16. This came about because when large amounts of cholesterol are present in the lipid, membranes formed shortly after the lipid is introduced into the chamber (as was the case for Fig. 16) initially have lower conductances than in the steady state following 30 min of equilibration (as was the case for Fig. 15). A slow dissolving of some cholesterol from the lipid into the aqueous phase is thought to be responsible for this effect. At lower lipid-cholesterol content (e.g., 36 and 52% of Fig. 16), such phenomena were unnoticeable

impossible for the macrotetralide molecules to move as carriers, we have prevented them from exerting their characteristic effects on the membrane.

Apart from the generally observed lower conductances in the presence of cholesterol, the membrane properties are qualitatively similar to those already described for cholesterol-free membranes. In particular, the direct proportionality between membrane conductance and concentration of monactin is still observed in the presence of cholesterol, as illustrated in Fig. 15. Also, the general behavior of the membrane conductance with increasing KCl concentration is seen to be similar in the presence and absence of cholesterol. This is illustrated in Fig. 16, where it is of some interest that the curves "level off" at increasingly lower ionic strengths as cholesterol is increased, indicating that the effects of ionic strength may depend on lipid composition.

Table 4. *The effects of the presence of cholesterol in the lipid on the electrical properties of bilayer membranes*[a]

Electrical property effected	Normal lipid (mho cm^{-2})	52 % cholesterol: 48 % normal lipid (mho cm^{-2})
G_{Na}	2.1×10^{-6}	9.4×10^{-8}
G_{Cs}	6.2×10^{-6}	2.2×10^{-7}
G_{Rb}	1.5×10^{-4}	7.4×10^{-6}
G_K	4.4×10^{-4}	1.7×10^{-5}
P_{Na}/P_K	0.0072	0.0074
P_{Cs}/P_K	0.023	0.026
P_{Rb}/P_K	0.47	0.45
G_{Na}/G_K	0.0048	0.0055
G_{Cs}/G_K	0.014	0.013
G_{Rb}/G_K	0.34	0.43

[a] All measurements were made in 10^{-2} M alkali chloride and 10^{-7} M monactin.

Despite the large effects of cholesterol on membrane properties described above and further illustrated in the upper portion of Table 4 (where membranes made from cholesterol-free lipid and a lipid containing 52% cholesterol are compared), the permeability ratios and conductance ratios are independent of the presence of cholesterol as can be seen in the lower portion of Table 4. Such an independence of the lipid composition of the ratios is expected theoretically if the complexes are "isosteric". This point will be examined in more detail in the Discussion.

Comparison of the Measured Membrane Properties with Those Predicted from Equilibrium Salt Extraction

Our results show that the membrane potential and conductance of bilayers are related to each other, and also that they depend on the composition of the aqueous solutions to which they are exposed, in the manner expected from the theory of paper I.

An even more stringent test of this theory is possible using Eqs. (6) and (7) together with the results of bulk equilibrium measurements of paper II to predict the permeability and conductance ratios of bilayer membranes. The extent to which the predictions of Eqs. (6) and (7) are fulfilled is seen by comparing the K_i/K_j ratios in the fifth column of Table 5 with the permeability and conductance ratios in the sixth and seventh columns. This is done graphically in Fig. 17 where the K_i/K_j ratios are compared to the permeability ratios on the left and to the conductance ratios on the right. A quantitative agreement for each of the macro-

Table 5. *The correspondence between bilayer membrane and salt extraction parameters* [a]

Macro-tetralide	Ion	K_i	$G_0(I)$ ($\times 2.08 \times 10^6$)	K_i/K_{Rb}	P_i/P_{Rb}	$G_0(I)/G_0(Rb)$
Nonactin	Li	0.05	0.077	0.00056	0.0021	0.00088
	Na	3.2	1.2	0.036	0.015	0.014
	K	190	190	2.1	2.1	2.1
	Rb	90	88	1.0	1.0	1.0
	Cs	11.5	7.1	0.13	0.077	0.082
	NH_4	9,000	580	100	—	6.7
Monactin	Li	0.10	0.23	0.00034	0.001	0.00073
	Na	8.0	4.4	0.028	0.015	0.014
	K	850	920	2.93	2.0	2.9
	Rb	290	310	1.0	1.0	1.0
	Cs	25	13	0.086	0.047	0.042
	NH_4	16,000	—	55.2	—	—
Dinactin	Li	0.15	0.48	0.00019	0.0014	0.00042
	Na	25	19	0.031	0.016	0.017
	K	2,000	2,300	2.5	2.4	2.1
	Rb	800	1,200	1.0	1.0	1.0
	Cs	46	31	0.058	0.033	0.027
	NH_4	24,000	—	30	—	—
Trinactin	Li	0.23	(0.011)	0.0002	(0.0018)	(0.000087)
	Na	42	(14)	0.036	(0.028)	(0.011)
	K	4,000	3,100	3.4	3.1	2.6
	Rb	1,170	1,200	1.0	1.0	1.0
	Cs	75	42	0.064	0.047	0.034
	NH_4	46,000	—	39	—	—

[a] Parenthesized values were obtained on imperfectly thinned membranes. K_i values are recalled from Table 15 of paper II. Bilayer data from Table 2, Fig. 4, and Fig. 8 for NH_4^+.

tetralide actins and for each cation is seen with the only significant exception of Li^+ for which the traces of NH_4^+ in our solutions was not negligible at the neutral pH of the bilayer measurements. [5]

The ability of Eqs. (1) and (6) to predict the bilayer membrane potential behavior is illustrated more explicitly in Fig. 18 where the experimentally observed values of membrane potential are compared to the theoretically predicted curves calculated taking the K_i/K_j values of Table 5 as permeability ratios.

5 The salt extraction equilibrium constants were determined at high pH where the effect of a given concentration of NH_4^+ is suppressed through reaction with OH^-.

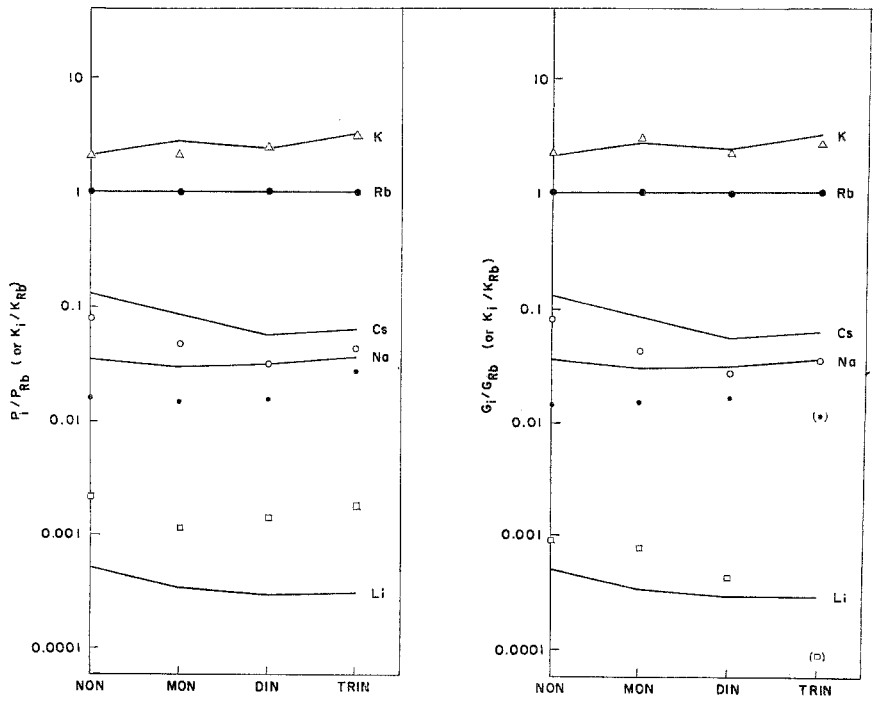

Fig. 17. Comparison of phospholipid bilayer permeability ratios and bilayer conductance ratios with bulk phase extraction ratios for each of the five alkali metal ions as a function of increasing number of methyl side groups for the macrotetralide actins. Left: Permeability ratios are plotted as points on the ordinate, and the solid lines connect the K_i/K_{Rb} values. Right: Same as left except that the points now indicate the ratio of the membrane conductances. Data tabulated in Table 5 were used

In addition to the above predictions involving only ratios, we have seen in Eq. (12) that for "isosteric" complexes a direct proportionality of the membrane conductance to the individual value of K_i is expected. That this is indeed observed is shown in Fig. 19 where the experimentally obtained points are seen to fall on straight lines of unit slope. This verifies that the membrane conductance, $G_0(I)$, is directly proportional to the salt extraction equilibrium constant, K_i, for all of the alkali cations. Furthermore, considering Fig. 20, we can see that the same proportionality constant, $A_i/B_i = 0.48 \times 10^{-6}$, suffices to relate membrane conductances to salt extraction equilibrium constants for all of the macrotetralide actin antibiotics as well as for all of the alkali cations.

Such a single proportionality indicates that, as a first approximation, the complexes have the same overall size and shape even when one, two or three methyl side groups are added to the nonactin molecule.

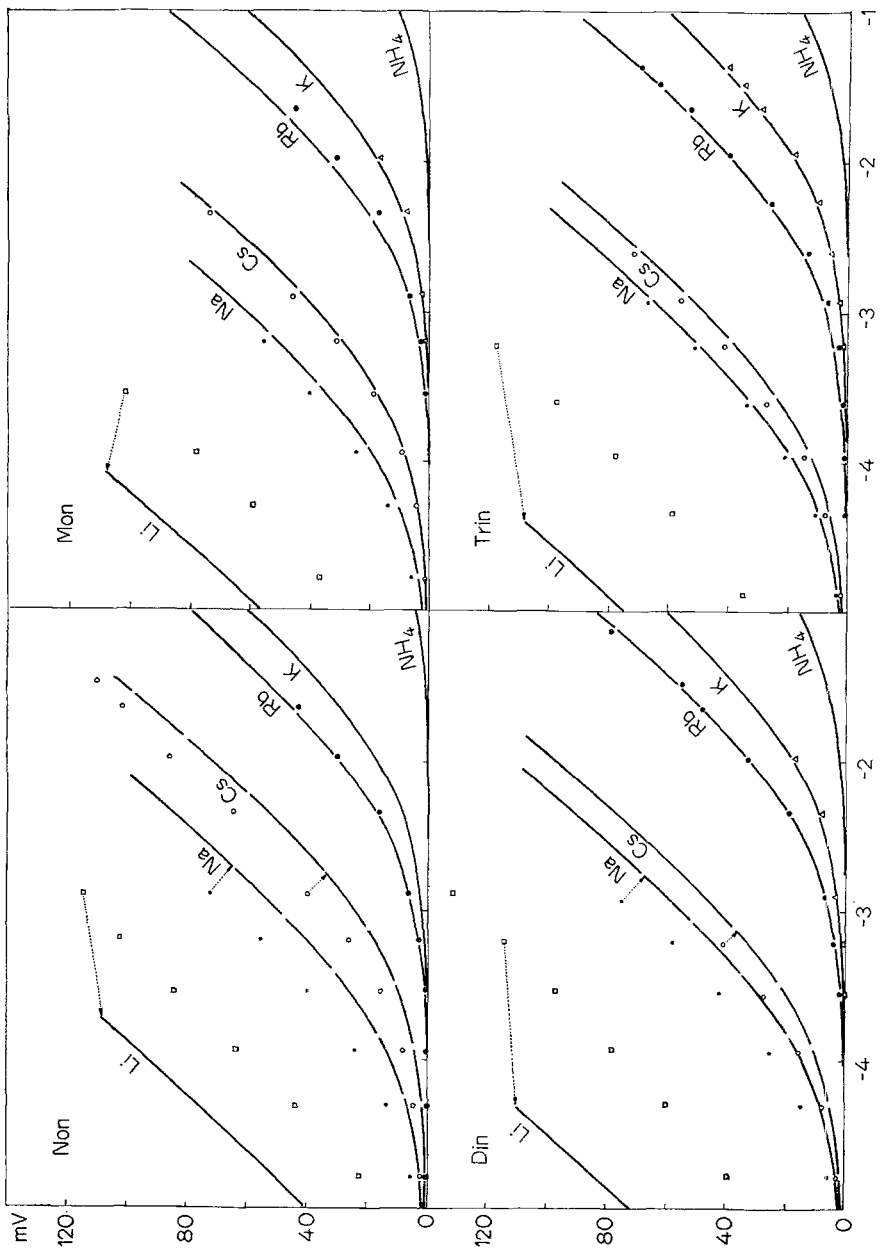

Fig. 18. Comparison between the predicted and observed membrane potentials for the macrotetralide actins. The solid lines are calculated from the expectations of Eq. (2), taking the K_j/K_K ratios of paper II as identical to the permeability ratios. The experimental points are from the data of Fig. 4

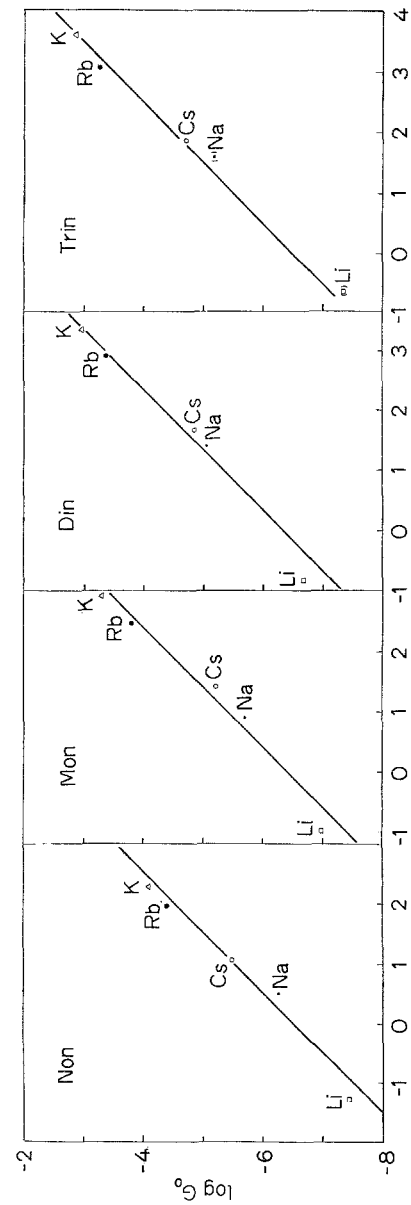

Fig. 19. The proportionality between the conductances of bilayer membranes and the equilibrium constants for salt extraction. Abscissa: Logarithm of the salt extraction equilibrium constants for the indicated macrotetralide antibiotics from paper II. Ordinate: Logarithm of membrane conductance measured at 10^{-2} M salt and the presence of 10^{-7} M macrotetralide concentration. (Data from Tables 2 and 5.) The lines of unit slope are drawn to indicate the proportionality expected from Eq. (12)

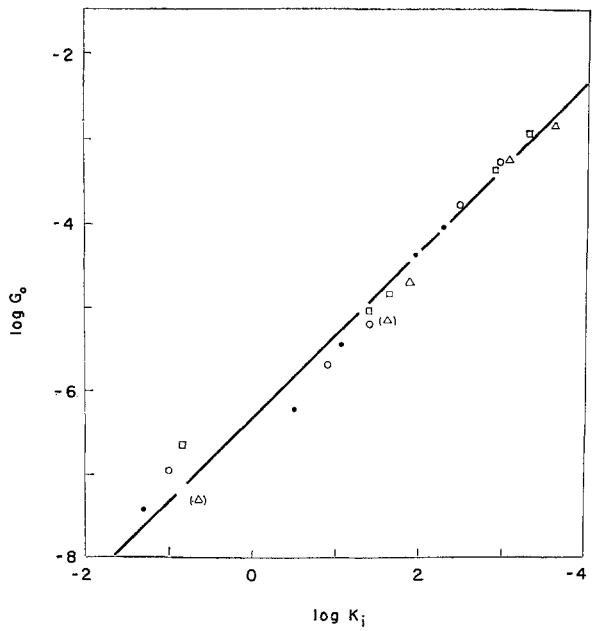

Fig. 20. The single proportionality between $G_0(I)$ and K_i. The data of Fig. 19 are condensed on a single log-log plot to show that a single proportionality constant relates the bilayer conductances $G_0(I)$ to the corresponding salt extraction equilibrium constants, K_i, for all of the macrotetralide actins and all cations. Filled circles represent nonactin; open circles, monactin; open squares, dinactin; and triangles, trinactin. The solid line of unit slope has an intercept of 0.48×10^{-6}, which is the value of the proportionality constant relating the conductances to the salt extraction equilibrium constants

Discussion

Further Evidence that the Complex is "Isosteric"

We have noted, in examining the data of Table 5, that the permeability ratios and conductance ratios are independent of the cholesterol content of the membrane. Recalling the definition of these ratios in Eqs. (2) and (4), we may write

$$\frac{P_j'}{P_i'} = \frac{G_0'(J)}{G_0'(I)} = \frac{u_{js}^{*'} k_{js}' K_{js}^+}{u_{is}^{*'} k_{is}' K_{is}^+} \tag{13}$$

for one lipid (*lipid'*); whereas

$$\frac{P_j''}{P_i''} = \frac{G_0''(J)}{G_0''(I)} = \frac{u_{js}^{*''} k_{js}'' K_{js}^+}{u_{is}^{*''} k_{is}'' K_{is}^+} \tag{14}$$

for the other lipid (*lipid''*). Note that only the parameters K_{is}^+ and K_{js}^+ are independent of the lipid (since they are defined as aqueous parameters in

paper I). The permeability ratio (or conductance ratio) for lipids of two different compositions are therefore expected to equal each other only if:

$$\frac{u_{js}^{*'}k_{js}'}{u_{is}^{*'}k_{is}'}=\frac{u_{js}^{*''}k_{js}''}{u_{is}^{*''}k_{is}''}. \tag{15}$$

The marked decrease in G_0 produced by adding cholesterol can be deduced from Eq. (3) to be a consequence chiefly of a change in the value of $(u_{js}^{*}k_{js})$ since the membrane thickness varies only imperceptibly upon addition of cholesterol [as judged by reflectance in our membranes and found by capacitance measurements by Hanai, Haydon & Taylor (1965*b*)], and all other variables in this equation are held constant in the experiment. Therefore, we know that the equality (15) holds despite large variations of the values of $u_{js}^{*}k_{js}$. This result is not generally expected since the k_{js}/k_{is} ratio should vary when the membrane composition is altered for complexes which are not "isosteric". It follows, however, for complexes that have the same size and shape regardless of the species of cation bound since for such "isosteric" complexes, as discussed in papers I and II:

$$\frac{u_{js}^{*'}}{u_{is}^{*'}}=\frac{u_{js}^{*''}}{u_{is}^{*''}}, \tag{16}$$

and

$$\frac{k_{js}'}{k_{is}'}=\frac{k_{js}''}{k_{is}''}=1, \tag{17}$$

so that condition (15) is satisfied.

Further indication that the complexes are "isosteric" comes from the observed equality of the bilayer permeability (and conductance) ratios to the ratios of salt extraction equilibrium constants measured in bulk systems. Such an identity is expected (*see* paper II) only if the mobility of the complex is independent of the particular species of cation sequestered, for only in this case does the mobility ratio drop out of Eq. (10, II). The single proportionality constant observed to relate membrane conductances and salt extraction equilibrium constants further substantiates this point. The above results for bilayers, taken together with the evidence of paper II for "isosteric" complexes in bulk phases, imply that the size and shape of the complex does not depend on the species of cation sequestered. The complex can therefore be visualized, in first approximation, as a large hydrophobic molecule bearing unit charge and having the same size for any of the alkali metal cations sequestered.

The Postulate that the Macrotetralide Actins are Carriers for Cations

The findings of the present paper, together with the evidence of paper II that the macrotetralide actin antibiotics form stoichiometric 1:1 complexes of the same size with the group Ia cations, strongly support the validity of the initial postulate of paper I that neutral antibiotics such as the macrotetralide actins produce their effects on lipid bilayer membranes by acting as molecular carriers of cations.

We are not the first to suggest a carrier mechanism of action for the macrocyclic antibiotics which indeed was proposed by Pressman, Harris, Jaeger, and Johnson (1967), by Lardy, Graven, and Estrado-O (1967), by Finkelstein and Cass (1968), by Tosteson (1968), by Wipf, Pache, Jordan, Zähner, Keller-Schierlein, and Simon (1969), and by ourselves (Eisenman et al., 1968; Szabo, Eisenman & Ciani, 1969). Pressman's and Lardy's suggestion was based on reasonable conclusions from molecular structure. In addition, Pressman argued by analogy from studies of the effects of antibiotics on salt extraction into model solvents. Other than our previous comparison of the properties of bilayers with those of a hexane bulk solvent (Eisenman et al., 1968), the most relevant data on the bilayer are Tosteson's (1968) studies of fluxes across membranes exposed to monactin-dinactin mixtures. From these, he has concluded that potassium ions cross the membranes singly and independently and that most of the potassium ions in the membrane are present as complexes.

Conclusions

Measurements of the effects of the macrotetralide actin antibiotics on the membrane potential and conductance of cholesterol-free lipid bilayers show that the observed properties are as expected from the theoretical model of paper I and the equilibrium constants measured in paper II. These results support the postulate that the macrotetralide actins exert their characteristic effects by solubilizing cations in the interior of the membrane as mobile charged complexes in accordance with the simple chemistry characteristic of their behavior in organic solvent phases.

In particular:

(1) The membrane potentials are found to be described by an equation of the Goldman-Hodgkin-Katz type for various cations with constant permeability ratios which are characteristic of the antibiotic molecule.

(2) The conductances of membranes interposed between identical solutions of a given alkali metal chloride are proportional to the aqueous concentration of antibiotic.

(3) The membrane conductance is also proportional to the aqueous salt concentration, provided that ionic strength is properly controlled. An ionic strength effect which mimics a "saturation" of conductance has been observed at salt concentrations higher than 10^{-3} M. Ionic strength probably affects the physical properties of the bilayer which leads to a reduced mobility of the complex at higher ionic strengths.

(4) For all of the macrotetralides, an equality between permeability and conductance ratios was found for each of the alkali metal cations, as was expected theoretically.

(5) To test further that it is reasonable to postulate the existence of a mobile complex, cholesterol was added to the lipid in order to decrease the mobility of the complex. A large decrease of membrane conductance was observed, as expected if the macrotetralides exert their effect as "carriers".

(6) It was also found that permeability and conductance ratios were the same, regardless of the cholesterol content of the lipid, as is expected if the overall size of the complex is the same for all cation species bound (i.e., the complex is "isosteric").

(7) A stringent test of the theory of paper I was made by using the equilibrium constants of salt extraction measured in paper II to predict both membrane potentials and conductances. The agreement between predicted and measured values indicates not only the validity of the theoretical approach but also supports the additional conclusion that the complex is "isosteric".

Overall Conclusion

The overall conclusion of this series of three papers is that, at least for simple molecules such as the macrotetralides, the theory of paper I can predict, using only the thermodynamic constants measured in bulk phases, the detailed electrical properties of bilayer membranes.

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