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Studies of the Conductance Changes Induced in Bimolecular Lipid Membranes by Alamethicin

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Summary. The addition of alamethicin to lecithin bilayers results in both voltage-dependent and voltage-independent conductance changes. In the voltage-dependent region, the slope of the conductance-voltage curve varies with the charge of the cation present in the aqueous phase. It may be shown that these effects may be accounted for by a kinetic model which incorporates the following suppositions: (1) alamethicin molecules are adsorbed at the membrane-water interface; (2) the effect of the potential is to redistribute alamethicin-cation complexes between the two surfaces of the bilayer; (3) conduction through the bilayer follows the surface interaction of approximately six alamethicin molecules; and (4) there is an assymetry in the rate constants for corresponding transitions on opposite sides of the bilayer.

The effects of alamethicin are found to be approximately the same at neutral and low pH and are unchanged when bilayers are formed from phosphatidyl serine rather than lecithin. These findings are discussed in relation to current hypotheses of the molecular nature of the conduction mechanism.

Early studies of bimolecular lipid membranes soon established that their electrical properties were passive in nature and indeed that their resistance was so high that the bilayer structure might fairly be regarded as an electrical insulator (Mueller, Rudin, Tien & Westcott, 1962). Thus, it was concluded that the permeability of the bilayer to ions was extremely low. In view of this major difference between natural and artificial membranes, considerable efforts have been made to induce ion transport properties and reduce the bilayer resistance to physiological levels by the incorporation of other molecules into the structure. The results of these investigations are contained in the authorative review of Mueller and Rudin (1969). These authors list over a dozen biological compounds which have a large effect (i.e., $>100\times$) on the bilayer resistance as well as a number of others which have a less marked effect.

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Among those molecules which are effective in lowering the bilayer resistance, the three compounds, alamethicin, monazomycin and excitability inducing material (EIM) are of particular interest in that they induce a conductance which is strongly voltage-dependent. In the case of alamethicin (Mueller & Rudin, 1968), the conductance is mainly cationic and increases with increasing voltage. Addition of basic protein to the system converts the conductance from cationic to anionic and introduces a negative resistance region into the current-voltage characteristic. Under suitable conditions this system displays most of the prominent electrical properties of the axon membrane.

How far the mechanisms of this artificial system resemble those of the natural membrane is at present unknown. Indeed, there is one obvious difference between the two systems, this being that the electrical properties of the artificial system are determined by the balance of cation and anion conductances as opposed to the well known sodium-potassium mechanism of the axon membrane (Hodgkin & Huxley, 1952). Nevertheless, the two systems share the important property of having ionic permeabilities, and hence conductances, which are dependent on the potential across the membrane. This property may reasonably be regarded as one of the most important single factors involved in nerve action. Because of the difficulties involved in studying the complex natural system, we have, at present, very little understanding of how such a voltage-dependent conductance arises. Thus, in our view there is much to be gained in using the artificial system to elucidate possible mechanisms by which this property might be produced in natural membranes.

Of the three compounds which induce voltage-dependent conductance in lipid bilayers, alamethicin is best characterized and hence appears the most promising material to study at present. Alamethicin is a cyclic polypeptide antibiotic produced by *Trichoderma viride* (Meyer & Reusser, 1967). The amino acid composition as recently determined by Payne, Jakes and Hartley (1970) is $(Ala)_2$(2-Methylalanine)$_7$(Glu N)$_2$$(Glu)_1(Gly)_1(Leu)_1(Pro)_2$ $(Val)_2$. This contains one 2-methylalanine residue less than the composition found by Meyer and Reusser (1967) and Reusser (1967). Payne *et al.* (1970) found slight variations in the amino acid composition, as is the case with many bacterial polypeptides. These authors have also determined the complete sequence of alamethicin. They find that the ring is closed by a peptide bond between the imino group of Pro 1 and the γ-carboxyl group of Glu 17 (Fig. 1). The 18th residue Glu N, is attached to Glu 17 by normal peptide linkage to form a side chain to the ring of 17 residues.

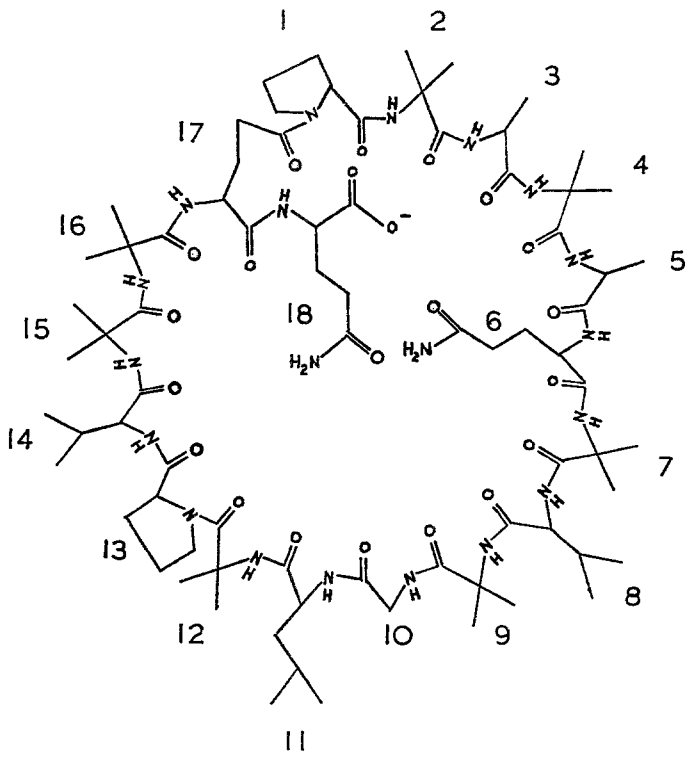

Fig. 1. Schematic structure of alamethicin (after Payne, Jakes & Hartley, 1970)

In the experiments reported here we have studied the effects of alamethicin on the electrical properties of lecithin bilayers. We have succeeded in reproducing most of the properties reported by Mueller and Rudin (1968), including the additional effects induced by the addition of protamine to the system. In the main, however, we have attempted to understand the effects of alamethicin alone, since we feel it is necessary to make progress in this direction before proceeding to examine the more complex situation resulting when protamine is also present in the system.

Materials and Methods

Egg yolk lecithin was extracted and purified by a method similar to that of Singleton, Gray, Brown and White (1965). The material was stored at −20 °C under nitrogen prior to use. Phosphatidylserine (PS) was obtained from Lipid Products and further purified on a silicic acid column according to the method of Long, Shapiro and Staples (1962). 1,2-dimyristoyl-L-phosphatidylcholine was synthesized by Mr. B. Partrick. The phospholipid samples gave single spots on thin-layer chromatography plates (silica gel H developed in chloroform-methanol-7*M* aqueous ammonia 230:90:15 v/v/v). The mem-

brane forming solution normally consisted of 1% lecithin in *n*-decane (Koch Light Puriss). The aqueous solutions were made up from 'Analar' grade reagents in distilled and deionized water. Alamethicin was obtained from the Upjohn Company and used without further purification.

The cell used for electrical studies was similar to that described by Mueller and Rudin (1969). Bilayers were formed by the brush technique across a 1-mm diameter hole in the side of a teflon pot. Electrical connections to the aqueous solutions on either side of the bilayer were made via KCl-Agar bridges and calomel electrodes. Current-voltage characteristics were measured by applying a dc voltage to the bilayer and measuring the current with a Keithley Model 417 Picoammeter. Potentials across the bilayer were measured with a Philbrick Nexus Operational Amplifier (input impedance $10^{12}\ \Omega$) and a Tektronix 502 A Oscilloscope. An additional circuit with a manual switch enabled potential pulses to be superimposed on the dc potential existing across the bilayer. All measurements were carried out at room temperature and neutral pH unless otherwise stated.

Normally, alamethicin was added to the inner chamber after the bilayer had formed. The variation of conductance with alamethicin concentration was determined by adding alamethicin in successive steps to the same bilayer. When the salt concentration was varied, measurements were made in a series of bilayers formed in solutions of different strengths.

Results

The current-voltage relationship for a lecithin bilayer in the presence of alamethicin is shown in Fig. 2*a*. The asymmetry which results from adding alamethicin to one side only of the bilayer persisted for the duration of an experiment (typically 10 to 20 min). When protamine was also added to the aqueous phase, the current-voltage curve was modified as shown in Fig. 2*b*.

In the presence of a KCl gradient across the bilayer, addition of alamethicin alone produced a potential which was positive on the side of low salt concentration indicating that the bilayer was relatively permeable to cations. The subsequent addition of protamine to the system reversed the sign of this potential. (Protamine in the absence of alamethicin had no effect.) By carefully controlling the amount of protamine added, it was possible to achieve a bistable situation in which the system could be switched between stable cationic and anionic resting potentials by the application of brief potential pulses. Fully developed action potentials however, were not observed.

According to Mueller and Rudin (1968) best results were obtained with membranes formed from lecithin-squalene 2:1 in decane. We have found that the presence of squalene had no observable effects on the results obtained in the experiments, although it appeared to assist in maintaining the stability of the membrane in the presence of alamethicin.

As reported in a previous preliminary communication (Chapman *et al.*, 1969) addition of alamethicin induces in the bilayer a voltage-independent

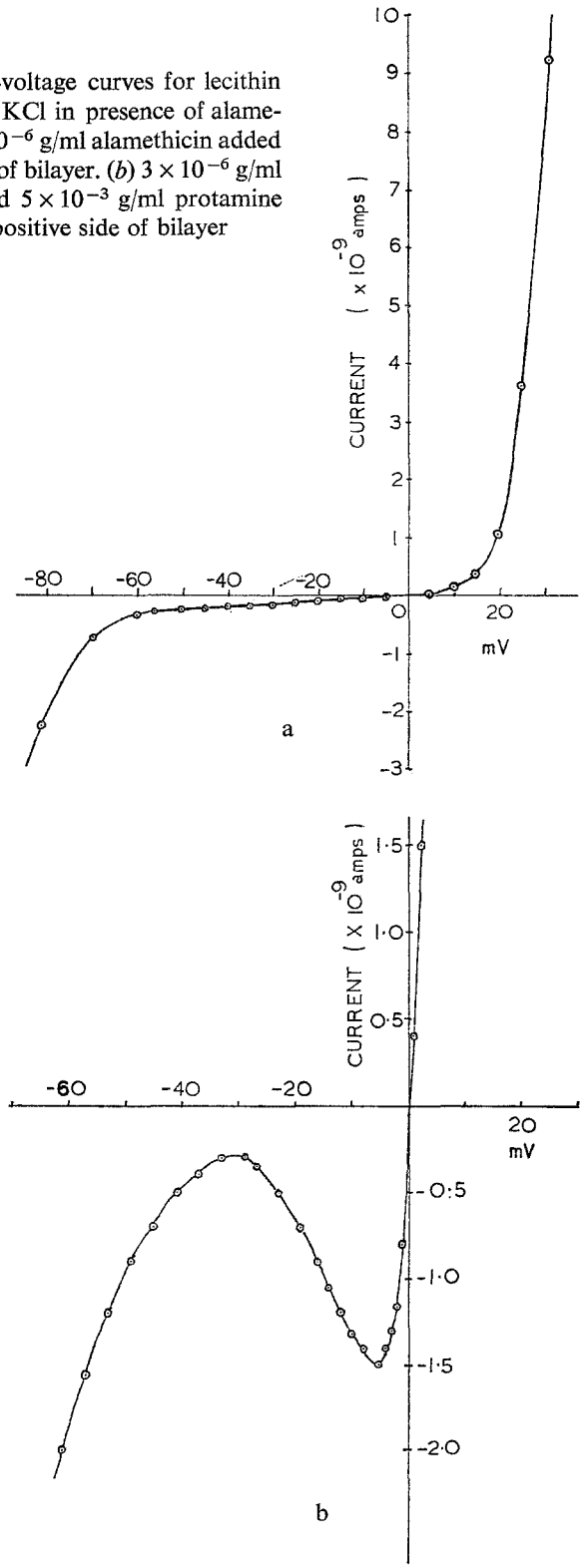

Fig. 2. Current-voltage curves for lecithin bilayer in 0.1 M KCl in presence of alamethicin. (*a*) 3×10^{-6} g/ml alamethicin added to positive side of bilayer. (*b*) 3×10^{-6} g/ml alamethicin and 5×10^{-3} g/ml protamine added to positive side of bilayer

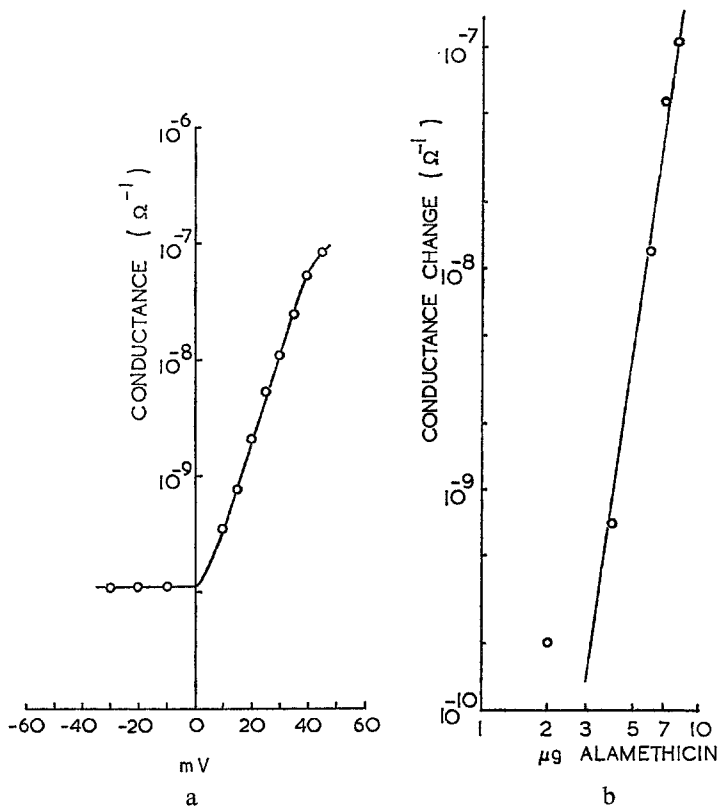

Fig. 3. (*a*) Typical conductance-voltage curve for lecithin bilayer. Aqueous phase 5 mM NaCl. 3 μg/ml alamethicin added to positive side of bilayer. (*b*) Variation of voltage-independent conductance with alamethicin concentration. Aqueous phase 100 mM KCl, volume of cell 3 ml. Potential 10 mV negative on side to which alamethicin is added

conductance change as well as the voltage-dependent effects. This may be seen in Fig. 3(*a*) which is a typical plot of log conductance against voltage. The voltage-independent conductance is always observed in the reverse direction of current (i.e., the side to which alamethicin is added is negative). The extent to which it penetrates into the forward direction is found to be somewhat variable. The variation of the voltage-independent conductance with alamethicin concentration is shown in Fig. 3(*b*). The results may be fitted to an expression of the form

$$\sigma = \sigma_0 + C(A_w)^r \tag{1}$$

where σ_0 is the intrinsic bilayer conductance, A_w is the alamethicin concentration in the aqueous phase and C and r are constants. Results obtained with different bilayers yielded values of r in the range 6 to 7 which is essen-

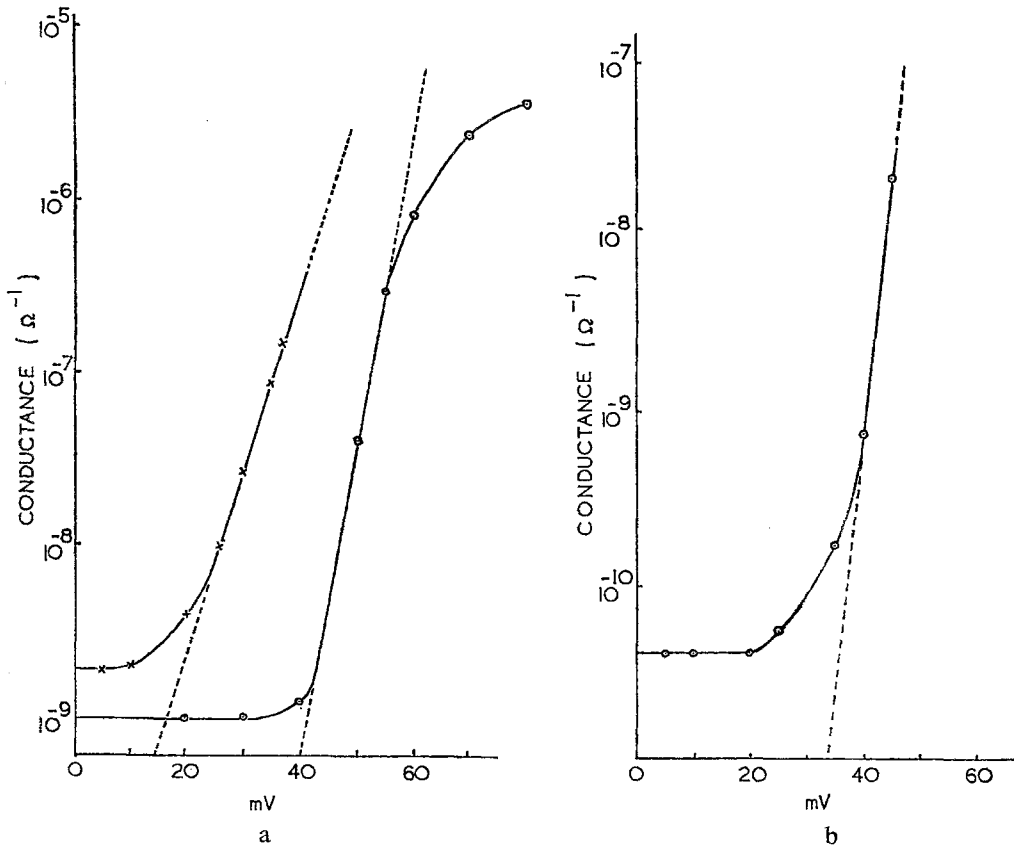

Fig. 4. Conductance-voltage curves for lecithin bilayer in the presence of various cations. Alamethicin added to positive side of bilayer. (*a*) ×—× 0.1 M KCl, 1×10^{-6} g/ml alamethicin; ∘—∘ 0.03 M $CaCl_2$ 0.5×10^{-6} g/ml alamethicin. (*b*) ∘—∘ 0.1 M $AlCl_3$ 0.5×10^{-6} g/ml alamethicin. The higher conductances include a small correction to take into account the series resistance of the circuit (4×10^4 Ω)

tially the same as the value $r = 6$ determined by Mueller and Rudin (1968) for the voltage-dependent conductance.

The dependence of the bilayer conductance on applied voltage in the forward direction in the presence of different cations is shown in Fig. 4. These results demonstrate a number of particular features. With some bilayers it was possible to increase the voltage to relatively high values without rupturing the bilayer. The conductance-voltage relationship was then found to become less steep at the higher voltages. This effect, which is illustrated by the $CaCl_2$ plot in Fig. 4, together with the voltage-independent conductance observed at low voltage, gives the whole curve a typical sigmoidal shape. Nevertheless, over a limited range the conductance varies approximately exponentially with voltage as described by Mueller and Rudin (1968).

By fitting this portion to the relation

$$\sigma \propto \exp \frac{nev}{kT} \tag{2}$$

(where n is a constant, e the electronic charge and v the applied voltage) values of n may be obtained.

For K^+, values of n were found in the range 4.5 to 6.5. When membranes were formed in $CaCl_2$ solutions the conductance-voltage curve became considerably steeper; values of n being in the range 10.5 to 12.5. Attempts were made to deduce a value of n for the trivalent ion Al^{+++}. Because of the extreme steepness of the conductance-voltage plot and the relative instability of membranes in the presence of this ion, it was difficult to deduce a satisfactory value. The value $n=17$ obtained from the curve shown in Fig. 4(*b*) should therefore be regarded as a rough estimate.

The conductance of bilayers in the presence of alamethicin was found to be independent of ion concentration in KCl solutions of 1 mM and above. With NaCl, however, the conductance did not saturate until an ion concentration of greater than 100 mM was reached. At lower ion concentrations the conductance decreased rapidly with decreasing ion concentration. In distilled water (salt concentration ~ 50 μM) alamethicin was ineffective in producing any conductance changes.

To investigate the importance of the free carboxyl group of alamethicin, some experiments were carried out at low pH. Under these conditions (aqueous phase 100 mM KCl, pH 2.0) it was necessary to add cholesterol to achieve sufficient stability (egg lecithin/cholesterol 1:1 mole ratio). It was found that the shape of the conductance-voltage curve at pH 2.0 was essentially identical with that at pH 7.0, although there was some increase in sensitivity of the bilayer to alamethicin at the lower pH.

Attempts to form bilayers from dimyristoyl lecithin were unsuccessful when the lipid was used alone. Eventually, it was found possible to form stable bilayers from a mixture of dimyristoyl lecithin/cholesterol 1:1 in *n*-decane: $CHCl_3$ 1:1 at 27 °C. With these bilayers the conductance-voltage relation was noticeably less steep than with egg lecithin (Fig. 5). The value of n calculated from Eq. (2) was about 3.0 to 3.5 for K^+ and about 7 for Ca^{++}. To check whether cholesterol was responsible for this lower value, some further measurements were made with egg lecithin-cholesterol bilayers in the same ratio of 1:1. These bilayers gave results which were indistinguishable from those obtained with egg lecithin alone.

In addition to the experiments with lecithin, some measurements were also made with bilayers formed from phosphatidylserine 1% in *n*-decane.

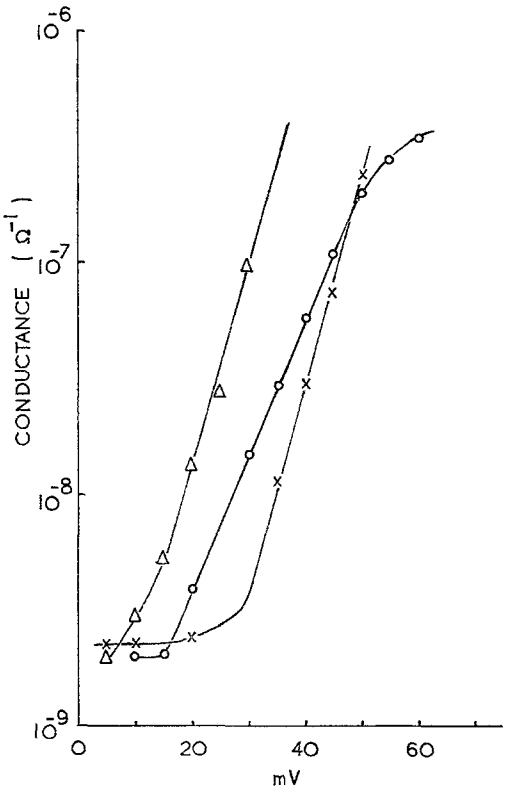

Fig. 5. Conductance-voltage curves obtained with synthetic dimyristoyl lecithin/cholesterol 1:1. 1.6×10^{-6} g/ml alamethicin added to positive side. ∘—∘ Aqueous phase 0.03 M KCl; ×—× Aqueous phase 0.03 M $CaCl_2$; △—△ Curve for egg lecithin/cholesterol 1:1 in 0.03 M KCl

The conductance-voltage curves obtained were, within the experimental uncertainty, indistinguishable from those obtained with lecithin bilayers under identical conditions (aqueous phase 100 mM KCl at pH 7.0, alamethicin concentration 0.5 μM in one set of measurements and 1.0 μM in a second set.)

Discussion

The Conductance-Voltage Curve

It is clear that alamethicin possesses structural characteristics common to other molecules such as the depsipeptide valinomycin, the actins and the polyethers which are capable of transporting ions across lipid membranes (Mueller & Rudin, 1967; Pressman, 1968). Thus, it is reasonable to suppose that cations can bind to alamethicin by ion-dipole interactions with peptide

carbonyls, while the high proportion of hydrophobic groups gives the complex lipid solubility. The complex effects observed in the bilayer when alamethicin is present, however, make it highly unlikely that alamethicin acts as a simple carrier. According to Mueller and Rudin (1968), monomers of the alamethicin-cation complex remain on the surface of the bilayer and are non-conducting. Application of a potential drives these monomers into the hydrocarbon region, where aggregation of six alamethicin molecules produces a conducting channel through the bilayer. This approach supposes that there is a causal relationship between the voltage dependence and the sixth-order dependence of conductance on ion and alamethicin concentration. The model which we propose below is also based on this supposition. It differs from the original model of Mueller and Rudin in that it emphasizes the surface active properties of alamethicin and gives more detailed consideration to the various transitions which may occur within and on either side of the bilayer. On this basis it is possible to give a satisfactory account of both the voltage-dependent and voltage-independent conductances.

Before discussing the model in more detail, we wish to draw attention to certain considerations which must be taken into account in any explanation of the voltage effects. In general terms, the voltage may be primarily regarded as causing a charge redistribution within the membrane; this in turn either directly or indirectly produces the conductance change. Such a charge redistribution could be produced, for example, by the motion of charged particles through the bilayer, by the rotation of dipoles or by bond polarization effects. Now the energy involved in this redistribution is from Eq. (2) and the experimental data about 6 eV in KCl solution. This is a relatively large amount of energy since it is equivalent to the electrostatic energy required to move 6 electronic charges across the bilayer against the applied potential. Alternatively, it is equivalent to the rotation of 6 dipoles each of moment ed/2 (where d is the bilayer thickness) from a parallel to an antiparallel position relative to the applied field. These conditions virtually rule out the possibility that the voltage effects result from a reorientation of a portion of the alamethicin molecule, such as the glutamine side chain. Further, the dependence of the energy involved on the valence of the cation makes it highly likely that the primary effect of the voltage is a redistribution of alamethicin-cation complexes.

We now consider how the redistribution of alamethicin-cation complexes within the bilayer may give rise to the observed conductance-voltage relationship. In our view, alamethicin readily enters a lipid bilayer even at zero potential. This is supported by the observation that alamethicin can transport cations from an aqueous to a butanol/toluene phase (Pressman,

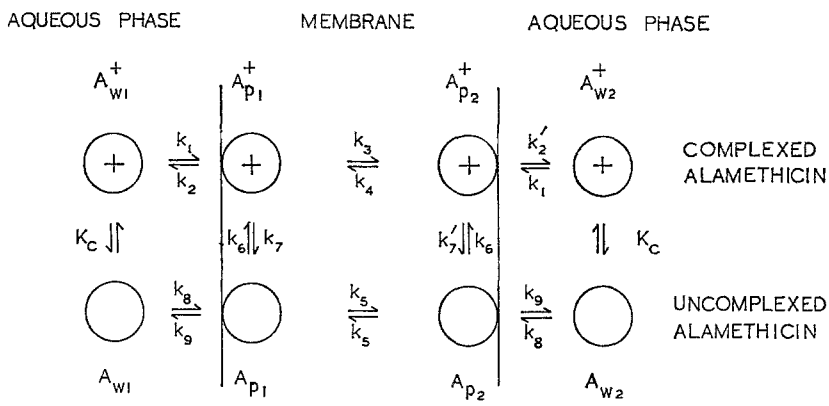

Fig. 6. Reaction scheme for alamethicin monomers in lipid bilayer. $[A_{w1}]$, $[A_{w2}]$ are the concentrations of alamethicin in the two aqueous phases and $[A_{p1}]$, $[A_{p2}]$ the concentrations in the two interfaces. The symbol + indicates complexed alamethicin

1968) and by the ability of alamethicin to penetrate lipid monolayers when added in nanomolar concentrations to the aqueous phase [Alamethicin alone has no effect on the surface tension at these concentrations.] (Chapman *et al.*, 1969). Within the bilayer it is probable that alamethicin molecules are situated at the membrane/water interface with their polar side chains in the aqueous phase. Evidence for this supposition comes from the high surface activity of alamethicin observed in monolayer studies and from microelectrophoresis measurements where alamethicin is found to impart a net negative charge to dispersed lecithin particles (Chapman *et al.*, 1969; Hauser, Finer & Chapman, 1970).

A general reaction scheme based on the above considerations is shown in Fig. 6. All rate constants are considered to be independent of voltage except for k_3 and k_4 which relate to transitions of alamethicin-cation complexes across the bilayer. Because of the very low intrinsic conductance of the bilayer the concentration of uncomplexed ions within the bilayer is assumed to be negligible. The general reaction scheme is somewhat complex since it includes association and dissociation both in the aqueous phase and at the membrane-water interface. To simplify matters, we consider the two limiting cases in which the rates of association and dissociation of alamethicin-cation complexes at the membrane surface are either fast or slow compared with the rates of adsorption and desorption.

Case 1: 'Slow' Surface Reaction. In this case we may consider the distribution in the bilayer of complexed and uncomplexed alamethicin separately. Further, since the conductance changes produced by alamethicin decrease

to zero with decreasing ion concentration, it is clear that only complexed alamethicin is involved in the effects. Thus, we need consider only the transitions (*see* Fig. 6)

$$[A^+_{w_1}] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [A^+_{p_1}]$$

$$[A^+_{p_1}] \underset{k_4}{\overset{k_3}{\rightleftharpoons}} [A^+_{p_2}]$$

$$[A^+_{p_2}] \underset{k_1}{\overset{k'_2}{\rightleftharpoons}} [A^+_{w_2}]$$

where $[A^+_{w_1}]$ and $[A^+_{w_2}]$ are the concentrations of the complex in the aqueous phase and $[A^+_{p_1}]$ and $[A^+_{p_2}]$ are the concentrations in the membrane-water interface on either side of the bilayer. Under the experimental conditions, alamethicin is added to one side only of the bilayer. This results in an asymmetry of the current-voltage curve which is maintained for the duration of the experiment. Thus, equilibrium between the two aqueous compartments is not re-established and $[A^+_{w_1}] \gg [A^+_{w_2}]$ at all times. However, since steady conductances are observed, we may suppose that the bilayer is in a quasi-equilibrium with the two aqueous phases such that $[A^+_{p_1}]$ and $[A^+_{p_2}]$ have steady values.

The conditions that $\frac{d[A^+_{p_1}]}{dt}=0$ and $\frac{d[A^+_{p_2}]}{dt}=0$ yield the equations

$$k_1[A^+_{w_1}]+k_4[A^+_{p_2}]=(k_2+k_3)[A^+_{p_1}] \tag{3}$$

$$k_3[A^+_{p_1}]+k_1[A^+_{w_2}]=(k'_2+k_4)[A^+_{p_2}]. \tag{4}$$

Adding these equations gives

$$k_1([A^+_{w_1}]+[A^+_{w_2}])=k_2[A^+_{p_1}]+k'_2[A^+_{p_2}]. \tag{5}$$

Solving for $[A^+_{p_1}]$ and $[A^+_{p_2}]$ we obtain (for $[A^+_{w_1}] \gg [A^+_{w_2}]$)

$$[A^+_{p_1}]=\frac{k_1(k'_2+k_4)[A^+_{w_1}]}{k_2k_4+k'_2(k_2+k_3)} \tag{6}$$

$$[A^+_{p_2}]=\frac{k_1k_3[A^+_{w_1}]}{k_2k_4+k'_2(k_2+k_3)}. \tag{7}$$

We now suppose that the conductance change results from an r-th order surface interaction of complexed alamethicin molecules. For convenience we use the term conducting 'channel' to describe the product of this interaction, although this should not be taken as implying any specific mechanism. The conductance σ is given by

$$\sigma=G_0K_H([A^+_{p_1}]^r+[A^+_{p_2}]^r) \tag{8}$$

where G_0 is the conductance of a single 'channel' and K_H is the equilibrium constant for 'channel' formation. (This equation assumes that 'channel' formation proceeds independently on the two sides of the bilayer.)

To derive the voltage dependence we write the rate constants k_3 and k_4 as

$$k_3 = A \exp \frac{(-E_3 + E_v)}{kT}, \tag{9}$$

$$k_4 = A \exp \frac{(-E_4 - E_v)}{kT} \tag{10}$$

where E_3 and E_4 are the energy barriers for the forward and reverse transitions across the bilayer at zero potential. E_v represents modification of these barriers by the potential and is given by $E_v = z\,e v/2$ (where z is the valence of the cation) if it is assumed that half the potential drop occurs at each interface.

The expression for the conductance is dependent on the relative magnitude of k_2'. For the condition that k_2' is negligibly small compared with k_2 and k_4 we obtain from Eqs. (6)–(10)

$$\sigma = G_0 K_H (K_{12} K_c [M^+] [A_{w1}])^r \left\{1 + \left(K_{34} \exp \frac{z e v}{kT}\right)^r\right\} \tag{11}$$

where $[M^+]$ is the concentration of cations in the aqueous phase, $K_{12} = k_1/k_2$, $K_{34} = k_3/k_4$ at $v = 0$, and K_c is the association constant for the reaction in the aqueous phase $[A_{w1}] + [M^+] \rightleftharpoons [A_{w1}^+]$. The form of the conduction-voltage curve calculated on the basis of Eq. (11) is given by curves *1* and *2* in Fig. 7. It may be seen that the equation successfully accounts for both the voltage-dependent and independent conductances and also correctly predicts that in both regions the conductance will depend on the same r-th power of the alamethicin concentration. In the voltage-dependent region $\left(\text{i.e., } \left[K_{34} \exp \frac{z e v}{kT}\right]^r \gg 1\right)$ the steepness of the conductance-voltage curve is proportional to z, thus accounting for the results with monovalent, divalent and trivalent cations. The value $r \approx 6$ may be deduced from the observed dependence of conductance on both voltage and alamethicin concentration, in good agreement with Mueller and Rudin (1968).

It should be noted that Eq. (11) represents the steepest conductance-voltage relationship that may be obtained with the present model. As k_2' becomes comparable with k_2 the relationship becomes less steep. This is illustrated by curves 3 and 4 in Fig. 7 which are calculated from the exact Eqs. (6)–(10) for $k_2'/k_2 = 0.1$ (with $k_2'/k_4 \ll 1$). Since the exact form of the

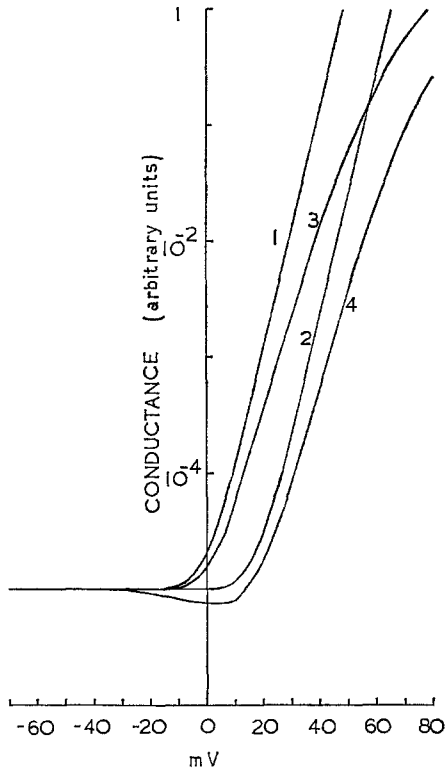

Fig. 7. Theoretical conductance-voltage curves. *Curve 1:* calculated from Eq. (11) with $K_{34}=1$, and $r=6$; *Curve 2:* as Curve 1 but $K_{34}=0.5$; *Curve 3:* calculated from Eq. (6)–(8) with $K_{34}=1$, $k_2'/k_2=0.1$, $k_2'/k_4 \ll 1$ and $r=6$; *Curve 4:* as Curve 3 but $K_{34}=0.5$. $z=1$ in all cases

measured conductance-voltage curve is somewhat variable it would be misleading to try to fit the theoretical curves to any particular set of experimental results. However, curves *3* and *4* appear to be more typical of the experimental data than curves *1* and *2*.

When k_2' is increased to the value $k_2'=k_2$ (i.e., the rate constants for alamethicin leaving the bilayer are the same on both sides) the model no longer predicts the observed conductance-voltage curve. This is most easily seen from Eq. (5) which for $k_2'=k_2$ gives $[A_{p1}^+]+[A_{p2}^+]=K_{12}([A_{w1}^+]+[A_{w2}^+])=$constant independent of voltage. Thus, in this case the voltage does not cause any increase in the total concentration of complexed alamethicin in the bilayer. Redistribution of alamethicin between $[A_{p1}^+]$ and $[A_{p2}^+]$ under these conditions can only increase the conductance by a factor of 2^6 (from Eq. (8) for $r=6$) whereas in practice changes of several orders of magnitude are observed.

Similar considerations to those above concerning k_2' apply equally to the model proposed by Mueller and Rudin[1] and to a number of other variations which we have examined. It appears likely to be a general requirement that there is some assymetry in the transitions which occur on either side of the bilayer. A possible physical explanation of this assymetry may be made as follows. When alamethicin molecules are adsorbed at the membrane-water interface on side 1 of the bilayer, they assume some specific orientation with respect to the surface. Due to the large size of the alamethicin molecule and the constraints of the lipid chains this orientation is maintained when alamethicin diffuses to the opposite side under the action of an applied potential. Thus, the orientation of the molecule with respect to the second surface is completely opposite to that at the first surface. The molecule may then have to reorient before desorption can occur resulting in an effective decrease in rate constant; alternatively, the misoriented alamethicin may desorb directly but at a relatively slow rate. This argument may also account for the fact that conduction by alamethicin monomers is not observed.

Case 2: 'Fast' Surface Reaction. In this case the rate at which alamethicin-cation complexes enter the bilayer is negligible compared with the rate of complex formation at the surface.

Thus the reactions which must be considered are:

$$[A_{w1}] \underset{k_9}{\overset{k_8}{\rightleftharpoons}} [A_{p1}]$$

$$[A_{p1}] \underset{k_5}{\overset{k_5}{\rightleftharpoons}} [A_{p2}]$$

$$[A_{p2}] \underset{k_8}{\overset{k_9}{\rightleftharpoons}} [A_{w2}]$$

$$[A_{p1}] + [M^+] \underset{k_7}{\overset{k_6}{\rightleftharpoons}} [A_{p1}^+]$$

$$[A_{p1}^+] \underset{k_4}{\overset{k_3}{\rightleftharpoons}} [A_{p2}^+]$$

$$[A_{p2}^+] \underset{k_6}{\overset{k_7'}{\rightleftharpoons}} [A_{p2}] + [M^+]$$

where $[A_{p1}]$ and $[A_{p2}]$ are the concentrations of uncomplexed alamethicin in the two surfaces. The conditions that $[A_{p1}]$, $[A_{p2}]$, $[A_{p1}^+]$, $[A_{p2}^+]$ are con-

1 Mueller and Rudin make the assumption that alamethicin monomers enter the hydrocarbon region of the bilayer from one side but cannot leave on the opposite side.

stant and $[A_{w1}] \gg [A_{w2}]$ yield the equations:

$$[A_{p1}]+[A_{p2}]=K_{89}[A_{w1}] \tag{12}$$

$$k_7[A_{p1}^+]+k_7'[A_{p2}^+]=k_6[M^+]K_{89}[A_{w1}] \tag{13}$$

$$k_4[A_{p2}^+]+k_6[M^+][A_{p1}]=(k_3+k_7)[A_{p1}^+] \tag{14}$$

$$k_8[A_{w1}]+k_7[A_{p1}^+]+k_5[A_{p2}]=(k_9+k_5+k_6[M^+])[A_{p1}] \tag{15}$$

where

$$K_{89}=k_8/k_9.$$

The solutions of Eqs. (12)–(15) for $k_7' \ll k_7$ are:

$$[A_{p1}^+]=K_{67}[M^+]K_{89}[A_{w1}], \tag{16}$$

$$[A_{p2}^+]=K_{67}[M^+]K_{89}[A_{w1}]\left(K_{34}\exp\frac{zev}{kT}+B\right) \tag{17}$$

where

$$B=\frac{k_7 k_5}{k_4(k_9+2k_5+k_6[M^+])}$$

and

$$K_{67}=k_6/k_7.$$

If B is small compared with $K_{34}\exp\frac{zev}{kT}$, substitution of $[A_{p1}^+]$ and $[A_{p2}^+]$ into Eq. (8) gives a conductance which has the same voltage dependence as that given by Eq. (11) in Case 1. Thus the experimental data do not enable us to distinguish between Cases 1 and 2 since under appropriate conditions either may give a good account of the conductance-voltage relationship.

The requirement $k_7' \ll k_7$ is the asymmetry factor already discussed under Case 1. A closely similar argument may be advanced to account for why the rate of complex dissociation should be different on the two sides of the bilayer. A recent structural model of alamethicin proposed by McMullen, Marlborough and Bayley (1971) may be relevant to this suggestion. This model is highly asymmetric with the most probable site of the cation located on one side of the molecule. An alamethicin-cation complex possessing this structure would clearly have to reorient after crossing the bilayer before the ion could be released.

The above analysis assumes in both cases considered that the alamethicin-cation complex carries a charge when crossing the bilayer. This will only be true if the cation is bound to the peptide carbonyls rather than the glutamine side chain of alamethicin and if the carboxyl group is undissociated as the complex crosses the bilayer. Further evidence that a charged group is not

essential for the observed properties is obtained from the finding that the effects of alamethicin are not significantly changed when the pH is reduced to 2.0 (i.e., below the pK of the carboxyl group). Pressman (1968) also finds that the ionization of the carboxyl group is not critical for complex formation.

The observation that the conductance saturates at a lower ion concentration in KCl compared with NaCl solutions indicates that alamethicin possesses some selectivity for K^+ over Na^+. [The available data suggest that the association constants differ by about an order of magnitude. This is somewhat greater than is indicated by bulk phase measurements (Pressman, 1968)]. This probably accounts for the assymetry in the current-voltage curve which is observed when equal concentrations of NaCl and KCl are on either side of the bilayer (Goodall, 1970).

Conducting 'Channels'

The above discussion makes no assumptions concerning the nature of the conducting 'channels' induced in the bilayer by alamethicin. Indeed the analysis would be equally valid if six alamethicin molecules aggregated to form a carrier. However, as pointed out by Mueller and Rudin (1968) a carrier mechanism appears unlikely in view of the size of such a complex. We have attempted to detect the existence of channels directly by observing changes in water permeability, but these experiments yielded complex and ambiguous results.

The simplest molecular model for alamethicin action supposes that the alamethicin rings are stacked one on top of each other through the bilayer to produce a conducting "pore" (Mueller & Rudin, 1968). This idea has been somewhat elaborated by Payne *et al.* (1970), who propose a similar structure stabilized by inter-molecular hydrogen bonds and with the polar side chain of each alamethicin molecule directed towards the center of the ring. Because of the flexibility of the side chain, a cation bound to the carboxyl group could be transferred from one molecule to the next by a "swinging arm" type of mechanism.

The experiments carried out with the shorter chain dimyristoyl lecithin might reasonably have provided some evidence relevant to the above model, since the number of alamethicin molecules needed to stack across the bilayer would be expected to depend on the bilayer thickness. In fact, the reduction we observe in the order of the reaction from approximately 6 to 3 is much greater than can be accounted for by the reduction in chain length from predominantly C_{18} for egg lecithin to C_{14}. The situation could be com-

plicated, however, if thinning effects of the type proposed by Parsegian (1969) were to occur. Attempts to measure an overall change in bilayer thickness in the presence of alamethicin using reflectance techniques (Cherry & Chapman, 1969) were not successful in detecting any such change.

Although there is little direct evidence for or against the stacked ring structure there are several factors which argue against it. First, McMullen (1970) has pointed out that a structure stabilized by intermolecular hydrogen bonding is not possible because of steric hindrance by the methyl group of the 2-methylalanine residues. Second, the model envisaged would produce a highly specific cation channel and it is rather difficult to see how this could be converted to an anion selective channel by interaction with basic protein. In our view, it is more likely that the 'channel' is relatively non-specific and selectivity arises from charged groups at the surface of the bilayer in the region of the 'channel'. Finally, as previously discussed, our measurements at low pH argue against the direct involvement of the carboxyl group.

The stacking model assumes that any lipid rearrangements that accompany the formation of the channel are of no more than secondary importance. A previous observation that the effects of alamethicin were largely independent of the particular lipid would appear to support this assumption (Mueller & Rudin, 1968). However, the present experiments with dimyristoyl lecithin demonstrate that the lipid can influence the conductance effects. Further, it is clear from studies with lipid dispersions that the addition of alamethicin causes considerable disorganization of the bilayer structure. (Chapman *et al.*, 1969; Hauser *et al.*, 1970).

Thus high resolution NMR measurements show that the lipid alkyl-chain signal is considerably broadened in the presence of alamethicin. With PS the effect occurs at a much lower alamethicin concentration than with lecithin. We have not observed any corresponding difference in conductance measurements with PS and lecithin films. This suggests that the structural changes in the lipid dispersion and the conductance effects in the film are not directly related, although this interpretation must be treated with some reservation since the actual alamethicin concentrations in the films are unknown.

Thus, no very definite conclusions about the molecular nature of the conducting 'channel' can be drawn at present. There is no evidence in favor of the stacking model and several reasons for rejecting it. Studies with lipid dispersions demonstrate the presence of alamethicin-induced phase changes; however, the idea that similar phase changes are involved in the conductance effects also lacks experimental support.

Axon Membrane

The well known equations of Hodgkin and Huxley (1952) provide an empirical basis for correlating the diverse electrical characteristics of the axon membrane. These authors pointed out that their equations could be given a physical basis if it was supposed that conductance changes in the membrane arose from the co-operative interaction of a number of charged particles which moved in response to changes in membrane potential. As they quite properly emphasized, this observation should not be regarded as providing any evidence that such a mechanism did in fact operate in the axon. The present model system studies also cannot give direct information about nerve action. Nevertheless, the close similarity between the proposed mode of action of the model system and the suggestion of Hodgkin and Huxley is indeed striking and must add credibility to the possibility of this type of mechanism occurring in the axon membrane. It has been recently proposed that low molecular weight proteins with strong aggregation properties occur as constituents of natural membranes (Laico, Ruoslahti, Papermaster & Dreyer, 1970; Tanner & Owens, 1971). If this claim is established, there would appear to be a distinct possibility that some of these molecules could have ion transport properties similar to those of alamethicin and other polypeptides.

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