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KINETICS AND STABILITY OF ALAMETHICIN CONDUCTING CHANNELS IN LIPID BILAYERS

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SUMMARY

It is already well-established that conduction in lipid bilayers containing alamethicin arises from the presence of complexes in which there are several molecules of the polypeptide. It is with the nature of these complexes that this paper is primarily concerned. While it is clear that increasing alamethicin concentration and increasing potential across the membrane favour their formation, the nature of the reactions involved has not yet been elucidated. Attempts have therefore been made to clarify the sequence of events leading to the establishment of a complex in its conducting state. It has been concluded that the most likely mechanism involves, initially, a non-field-dependent aggregation of the alamethicin, in the plane of the membrane, into non-conducting oligomers. These then appear to undergo movement normal to the membrane (which is field dependent) to form the conducting species. Temperature studies have shown that the various conducting states of the oligomer have effectively equal enthalpies, and that the activation energies for transitions between these states are all approx. 1.2 kcal/mol. The corresponding rate constants are very sensitive to the lipid composition of the membrane and a variety of different systems has been examined in order to clarify the origins of this effect. The only conclusion from this part of the work is that lipid fluidity might be involved.

INTRODUCTION

It is now well-established that alamethicin facilitates the passage of both anions and cations across lipid bilayers [1–5] and that the membrane conductance is a strong function of the applied potential [1, 3, 5, 6]. It is also known that the polypeptide forms pores having weak ion selectivity [2, 3, 5, 7]. There are, however, still several outstanding problems concerning its mechanism of action. One of these concerns the origin of the different conductance levels which are discernible within a single current pulse (Fig. 1). The levels appear to arise from an array of interacting channels [4, 5], and it has been concluded that several alamethicin molecules combine together to

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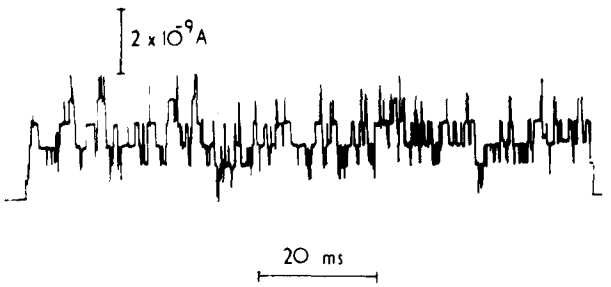

Fig. 1. A single current pulse for a glycerol monooleate-cholesterol-hexadecane membrane containing alamethicin in 2 M KCl. The monoglyceride and cholesterol were, respectively, 6 and 14.8 mM in the hexadecane, and the alamethicin was approx. 10^{-7} M in the aqueous phases on one side only of the membrane. The applied potential was −220 mV, the alamethicin compartment being negative.

form these multi-channelled complexes (oligomers). It is nevertheless not clear whether the different conductance levels arise (a) from conformational or positional changes within the preformed oligomers, or (b) from variations in the number of molecules involved in the oligomer.

Another unsolved problem is the origin of the large variations in the frequency of transitions between the conducting level of the oligomer, which are observed in membranes of different lipid composition. In this paper these and related questions have been examined by analysing the fluctuations in membrane current found under a variety of conditions of temperature, applied potential, membrane thickness, lipid chain unsaturation, etc. Some reasons have been found for preferring mechanism (a) described above and some possible explanations for the variation in transition frequency have been eliminated.

THEORY

Boheim [8] and Baumann and Mueller [14] have presented analyses of the opening and closing of the alamethicin channels based on model (b) described in Introduction. From a consideration of arguments such as those given recently by Hall [15], and in anticipation of the conclusions which will be drawn later, the treatment given below is based on model (a). Each alamethicin-conducting complex has been supposed to enter and leave different energy states corresponding to the various possible levels of conductance. Thus, if there were a large number N of independent complexes, then the number N_i in energy state i is related to the number N_j in state j by the expression

$$-\Delta G_{ij}^{\ominus} = RT \ln \frac{N_i}{N_j} \tag{1}$$

where $\Delta G_{ij}{}^{\ominus}$ is the standard free energy difference between the two states.

If only one complex is available for study then, over a sufficiently long time period of conduction, T_{total} (T is such that a greater time period would not affect the outcome), the time, T_i for which the unit is in any state i, is proportional to the number N_i in the former distribution where N units were available, i.e.

$$\frac{T_i}{N_i} = \frac{T_j}{N_j} \tag{2}$$

Therefore,

$$-\Delta G_{ij}^{\ominus} = RT \ln \frac{T_i}{T_j} \tag{3}$$

Thus, in the set time period, T_{total}, the times T_i, T_j, etc. are related to the various free energies of the unit and these are independent of the frequency of entry into the available states.

The mechanism which controls the transitions between states is assumed to restrict these changes to a linear chain of events, i.e. the ith conductance level can only be entered from the $(i-1)$th or the $(i+1)$th levels. The conducting pulse therefore begins and ends with the first level. All the transitions appear to follow first-order kinetics. The evidence for this is given below and is obtained from the analysis of the durations in any particular state. If transitions are first order, then the number of times, $n_{i,t}$, that the ith state has a duration greater than t (in the sufficiently large set time period of conduction, T_{total}) is given by

$$n_{i,t} = n_{i,o}\, e^{-k_i t} \tag{4}$$

where $n_{i,o}$ is the total number of times the ith state is entered, and k_i is the composite rate constant for the ending of the ith state, i.e.

$$k_i = k_{i\uparrow} + k_{i\downarrow} \tag{5}$$

In histogramming it is normally more convenient to distribute durations between compartments of time limits t and $t+\delta t$ and Eqn. 4 is replaced by

$$\frac{\delta n_{i,t}}{\delta t} = -k_i n_{i,o} e^{-k_i t} \tag{6}$$

where $\delta n_{i,t}$ is the number of durations with magnitudes between t and $t+\delta t$. It will be shown that plots of $\ln \delta n_{i,t}$ versus t are close to linearity and that $k_{i\downarrow}$ and $k_{i\uparrow}$ are therefore either unimolecular rate constants or that there is a peculiar compensation between them which gives rise to a pseudo first-order system, a situation which seems rather unlikely.

From the mechanism controlling the allowed changes between states it is a natural consequence that there must be an equal number of forward and reverse transitions. The rate at which a unimolecular process proceeds depends on the concentration (in this case, it is the fraction of unit time spent in, rather than the fraction of the number of identical units per unit volume or area in, a particular state; see Eqn. 2) and on the rate constant of the process. The mean life-time of the ith state is $1/k_i$ and thus the fraction of unit time spent in the ith state is $n_{i,o}/k_i T_{\text{total}}$. The frequency, $f_{i/(i+1)}$ of the transitions between ith and $(i+1)$th states is therefore

$$f_{i/(i+1)} = \frac{n_{i,o}}{k_i T_{\text{total}}} k_{i\uparrow} \tag{7}$$

and, by a similar argument,

$$f_{(i+1)/i} = \frac{n_{(i+1),o}}{k_{i+1}\, T_{\text{total}}}\, k_{(i+1)\downarrow}$$

$$= f_{i/(i+1)}$$

The values of $n_{i,o}/k_i T_{\text{total}}$ and $f_{i/(i+1)}$ are determined experimentally and, from Eqn. 7, the simple rate constants $k_\uparrow$ and $k_\downarrow$ for the transitions can be evaluated.

The question now arises as to whether or not the parameters associated with any particular conductance level depend on the previous history of the conducting aggregate. More specifically, do the values of $k_{i\downarrow}$ and $k_{i\uparrow}$ (parameters which are associated with the stability of the ith state) depend on which of the two states (the $(i+1)$th or the $(i-1)$th) the unit was in previously? Double valued parameters of this kind could arise if, for example, after a change in conductance, thermal energy had not sufficient time to equilibrate before the occurrence of the next transition. The measured values of $k_{i\uparrow}$ and $k_{i\downarrow}$ would then be composites depending on the relative frequencies of entry to the ith from the $(i+1)$th and $(i-1)$th states. For conducting levels of relatively long duration, $k_{i\uparrow}$ and $k_{i\downarrow}$ appear to be univalued, but it is conceivable that this may not be true for transitions from states which have had an extremely brief life-time.

MATERIALS AND METHODS

Materials

The alkane solvents and the hexadecyl bromide were obtained from Koch-Light Laboratories. The former were all "puriss" grade while the latter was "purum". In each instance the solvents were passed through alumina columns before use.

The glycerol monooleate and the glycerol monopalmitoleate were obtained either from Sigma or from Nu Chek Prep Inc. (Minn.) and were confirmed to be $\geqslant 99\,\%$ pure monoglyceride by gas-liquid and thin-layer chromatography. For forming "black" films they were used at 5 mM in the above solvents.

The phospholipids, bacterial phosphatidylethanolamine (Supelco, Inc., Bellafonte, Pa., U.S.A.), 1,2-dioleyl-*sn*-glycero-3-phosphorylcholine (kindly supplied by Dr. J.C. Metcalfe, Department of Pharmacology, Cambridge), and brain phosphatidylserine (Lipid Products) were all analysed and have been described in detail elsewhere [9, 10]. They were used at 1 % (w/v) in the non-polar solvents. The cholesterol was of Biochemical Standard grade from B.D.H. Chemicals Ltd.

Aqueous solutions, prepared from doubly distilled water and Analar grade reagents, were normally unbuffered and were approx. pH 5.4–5.6. In the study of the effects of pH, Tris and acetate buffers were used at concentrations below 0.2 M.

Methods

The apparatus for forming and examining the "black" lipid films was essentially as described by Hladky and Haydon [11]. The films were formed in the horizontal plane and were made as small as possible (usually approx. 0.1 mm diameter) in order to keep electrical noise to a minimum. The current fluctuations were first recorded on a tape recorder (SE Data 2600) and subsequently either transferred to a chart recorder or analysed directly using a Biomac 500 (Data Laboratories Ltd., London) so as to yield the frequencies $f_{i/(i+1)}$ and the times T_i. Good oscilloscope records for

the faster systems are not easy to obtain owing to the limitations of the storage facility (see e.g. ref. 4, Fig. 3). However, a more convincing demonstration than Fig. 1 of the resolving power of the equipment is given by the tape/pen recorder trace in Fig. 11 of ref. 16. The accuracies of the quantities T_i and k_i depend on i. Thus, the response time of the amplification system, which also clamps the voltage across the film, limits the accuracy with which the duration T_i of any particular state may be measured, and the percentage accuracy will obviously increase as the duration increases. Hence, when i is very small or very large and T_i is small the errors will be maximal. With the exception of the results for the terminal i values, T_i and the rate constants should be reliable to two significant figures.

RESULTS

Occurrence of the current pulses

The time intervals between the current pulses (one of which is shown in Fig. 1) have a continuous range of values, and it is found that the frequency of occurrence of given intervals decays exponentially with the length of the interval (Fig. 2). This observation suggests that, under given conditions, the probability of occurrence of the current pulses is constant, i.e. that the pulses are random events. It has been shown elsewhere [5] that the probability of occurrence of the pulses increases rapidly with the applied potential and that this is the main cause in these systems of the very strong

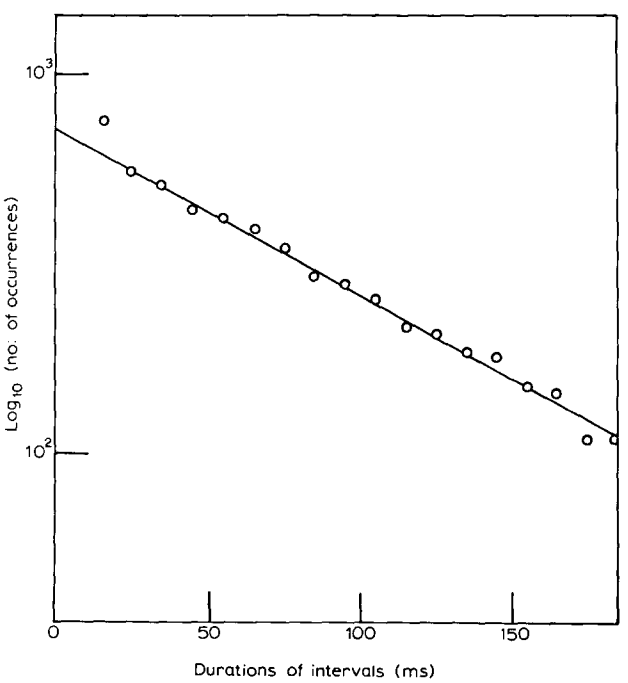

Fig. 2. The variation of the occurrence of current pulses with the duration of the interval between them. The membrane and aqueous phase was as for Fig. 1. The alamethicin concentration was approx. $5 \cdot 10^{-8}$ M and the applied potential was 150 mV.

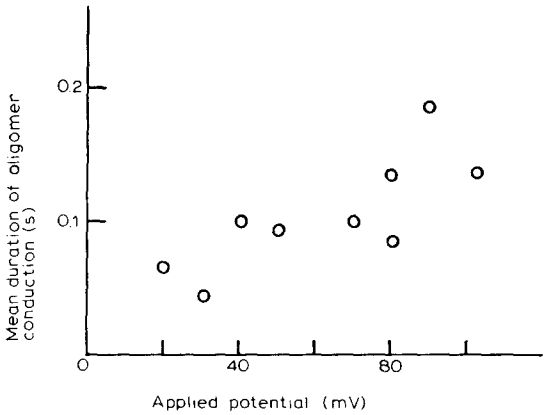

Fig. 3. The variation of mean duration of conducting oligomers as a function of membrane potential. The lipid membrane and the aqueous solution were as for Fig. 1.

dependence of membrane conductance on potential.

The durations of the pulses themselves may be analysed in the same way as the intervals between them and, here also, it is found that the frequency of occurrence declines exponentially with the length of the event [5]. In contrast to the intervals, however, the pulse durations are only weakly dependent on the applied potential. Fig. 3 illustrates this for systems in which the frequencies of appearance of pulses were similar.

Obviously no relation between the lengths of the intervals and the duration of the pulses would be detectable unless one oligomer were to spend an appreciable fraction of the total time in the conducting state.

Transitions between levels within pulses

The results presented below are all concerned with the fine structure of the current pulses and, specifically, with the kinetics of the transitions between the various conductance levels (Fig. 1). The data have mostly been obtained for systems in which the alamethicin has been present on the negative side of the membrane (this condition will be referred to later simply as "negative applied potentials"). As will be seen from the tables, the rate constants, obtained as described in Theory, exhibit clear trends. Thus, for the opening of the channels, the constants normally decrease as the conductance level increases, while the reverse is found for the closing process. These patterns give rise to a variation in the stability of the levels (a measure of which is T_i/T_{total}) which has a maximum slightly below the midpoint of the range (which can be up to nine levels). Similar results are obtained for the case in which the electric field is reversed (i.e. positive applied potentials) but the conduction level of maximum stability is lower and the number of levels is smaller, usually terminating in the fifth. Very infrequently a sixth level may also be observed.

The opening and closing rate constants for the individual channels have been calculated from plots such as those of Fig. 4. While greater accuracy of the data would be advantageous (especially for the low and high levels) the plots are consistent with the rate constants being first order.

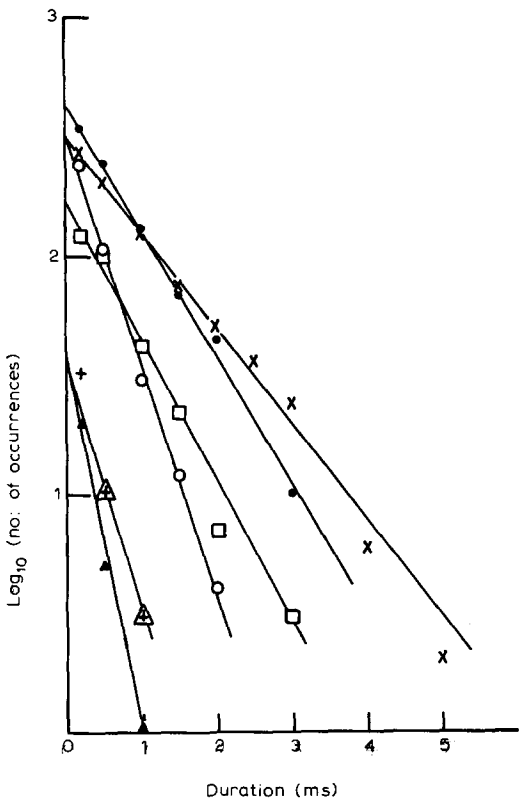

Fig. 4. The frequency of occurrence, in a membrane of glycerol monooleate (6 mM)+hexadecane+cholesterol (14.8 mM) of alamethicin-conducting states, i, having durations between defined limits (see Eqn. 6). $i = 1$, ▲; 2, +; 3, ○; 4, ●; 5, ×; 6, □; 7, △. The aqueous solution was 3 M KCl and the applied potential −300 mV.

Temperature dependence. Table I contains thermodynamic and kinetic data for a range of temperatures. An obvious feature of the results is the constancy of T_i/T_{total}. From Eqn. 3,

$$-\frac{\Delta G_{ij}^{\ominus}}{T} = -\frac{\Delta H_{ij}^{\ominus}}{T} + \Delta S_{ij}^{\ominus} = R \ln \frac{T_i}{T_j}$$

Assuming $\Delta H_{ij}^{\ominus}$ and $\Delta S_{ij}^{\ominus}$ to be independent of temperature, it follows that

$$R \frac{\mathrm{d}}{\mathrm{d}T} \ln \frac{T_i}{T_j} = \frac{\Delta H_{ij}^{\ominus}}{T^2}$$

If an estimated error of approx. 10 % is allowed in the T_i values the experiments suggest that $\Delta H_{ij}^{\ominus} = 0 \pm 1$ kcal/mol, i.e. that all the conducting states of the oligomer have the same enthalpy.

The rate constants for the opening and closing of the channels are, however, functions of the temperature (Figs. 5a and 5b). The interpretation of these plots in

TABLE I

THE EFFECT OF TEMPERATURE ON THE ALAMETHICIN-CONDUCTING STATES

System: phosphatidylethanolamine-decane/2 M KCl: applied potential, 150 mV.

(A) Fraction of time (T_i/T_{total}) spent in each conducting state during the period of conduction

	1*	2	3	4	5
18 °C	0.24	0.48	0.23	0.042	0.001
33 °C	0.30	0.41	0.21	0.048	0.002
50 °C	0.23	0.49	0.25	0.029	0.001

(B) Thermodynamic parameters for the ith, relative to the first, conducting state at 18 °C (kcal/mol)

$\Delta G_{ij}^{\ominus}$	0	−0.40	0.025	1.0	3.2
$\Delta H_{ij}^{\ominus}$	0	0	0	0	0
$T\Delta S_{ij}^{\ominus}$	0	0.40	−0.025	1.0	−3.2

(C) Rate constants for the opening (↑) and closing (↓) of channels (s^{-1})

	$k_{1\uparrow}$	$k_{2\uparrow}$	$k_{3\uparrow}$	$k_{4\uparrow}$
18 °C	27	12	7.8	7.0
33 °C	71	42	25	21
50 °C	139	66	51	65
	$k_{2\downarrow}$	$k_{3\downarrow}$	$k_{4\downarrow}$	$k_{5\downarrow}$
18 °C	14	25	43	280
33 °C	52	81	110	600
50 °C	64	130	440	1400

* '1' denotes first conducting level; '0' would correspond to no conduction.

terms of the Arrhenius equation shows that the activation enthalpies for the opening and closing processes are similar and, in magnitude, approx. 1.2 kcal/mol.

Potential dependence. Although the macroscopic conductance of alamethicin in lipid bilayers is an exponential function of the applied potential, increasing approx. 10-fold for every 10 mV rise, the conductance levels within a unit pulse are only slightly potential dependent. Fig. 6 shows the fraction of the total conducting time the oligomer spends in various conducting states. As can be seen, the higher states become more stable and the lower states less stable as the potential increases. The behaviour of the individual rate constants is presented in Table II. Except for the extreme values which, owing to the relatively small number of measurements that can be made, tend to be inaccurate, the constants decrease with increasing potential, a pattern which was observed for both positive and negative fields and which was independent of the nature of the lipid.

Unfortunately, the significance of the data in Table II is obscured by the fact that in order to obtain clear single pulse records over a range of potential it was necessary to vary concomitantly the alamethicin concentration. It is not obvious, therefore, that the variation in the rate constants is a consequence of the potential alone, as it could be affected also by the amount of the polypeptide present in the membrane. This ambiguity exists only because it has not yet been settled which of the

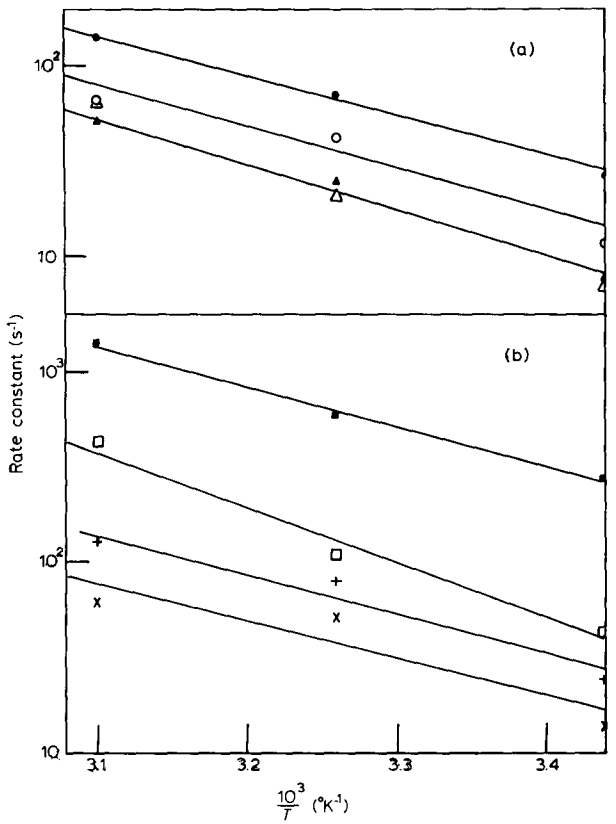

Fig. 5. The rate constants for the opening and closing of alamethicin channels as a function of temperature. The membrane was phosphatidylethanolamine+decane in 2 M KCl and the applied potential 150 mV. (a) Opening rate constants for transitions: 1, ●; 2, ○; 3, ▲; 4, △. (b) Closing rate constants for the transitions: 2, ×; 3, +; 4, □; 5, ■.

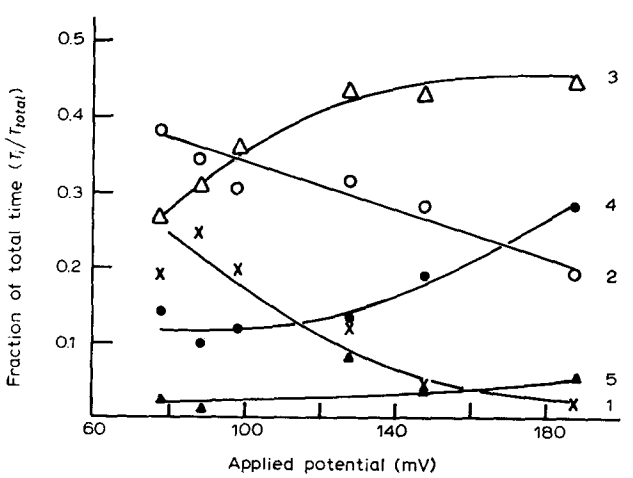

Fig. 6. The relative stability of conducting levels (*i*) as a function of membrane potential (see Eqn. 3). The membrane was glycerol monooleate (6 mM)+cholesterol (14.8 mM)+decane and the aqueous phase 2 M KCl.

TABLE II

THE EFFECT OF MEMBRANE POTENTIAL ON THE CONDUCTING STATES OF THE ALAMETHICIN OLIGOMER

System: Glycerol monooleate (6 mM)-cholesterol (14.8 mM)-decane/2 M KCl.

(A) Fraction of time spent in each conducting state during the period of conduction

	State					
	1	2	3	4	5	6
−150 mV	0.07	0.23	0.36	0.25	0.07	0.005
−223 mV	0.04	0.18	0.47	0.24	0.04	0.009

(B) Rate constants for the opening (↑) and closing (↓) of channels (s^{-1})

	$k_{1\uparrow}$	$k_{2\uparrow}$	$k_{3\uparrow}$	$k_{4\uparrow}$	$k_{5\uparrow}$
−150 mV	610	630	360	170	160
−223 mV	1300	450	210	83	200
	$k_{2\downarrow}$	$k_{3\downarrow}$	$k_{4\downarrow}$	$k_{5\downarrow}$	$k_{6\downarrow}$
−150 mV	190	400	500	620	2000
−223 mV	280	170	400	480	970

two models described in Introduction are correct. Thus, if the monomeric polypeptide molecules first aggregate into the oligomers, which are then driven into a conducting state by the applied field (model a), the applied potential must act only by favouring particular conformational states of the oligomer and no effect of the alamethicin concentration would be expected. If, on the other hand, the monomeric alamethicin is first driven into the membrane by the field, where it then aggregates to varying degrees, each of which, corresponds to a different conducting level (model b), then both the applied potential and the alamethicin concentration could be important. In an attempt to distinguish between the two models, small voltage steps were super-

TABLE III

THE DEPENDENCE OF SINGLE CHANNEL EQUILIBRIA AND RATE CONSTANTS ON THE MEMBRANE POTENTIAL, AS DETERMINED BY THE SINGLE PULSE METHOD (see text)

System: Glycerol monooleate (6 mM)-cholesterol (14.8 mM)-hexadecane/2 M KCl.

	110 mV	120 mV
T_3/T_2	0.50	0.76
T_4/T_3	0.20	0.17
$k_{2\downarrow}$	120	160
$k_{2\uparrow}$	100	170
$k_{3\downarrow}$	200	220
$k_{3\uparrow}$	58	99
$k_{4\downarrow}$	280	570
$k_{4\uparrow}$	51	100

imposed on a constant background voltage during the conduction of a single current pulse and the rate constants determined before and after the voltage changes. During long pulses at 115 mV applied, a square wave of period 0.2 s and amplitude 5 mV (giving a total variation of 10 mV) was superimposed. The first one-third and last two-thirds of each half wave were analysed. The results are given in Table III. Care was taken to ensure that the period of the superimposed square wave was longer than the relaxation time of macroscopic conductance following the same voltage step.

The influence of membrane thickness and of lipid composition. It has already become clear that while the lipid composition of a membrane may not appreciably affect the fraction of time which an oligomer spends in the various conducting states, the rate constants for the transitions between the states may be very profoundly affected [3, 7]. One of the membrane properties particularly influenced by the lipid composition is the thickness, and it is of interest to see how the rate constants vary when this factor specifically is changed. The relevant data are given in Tables IV and V. As can be seen, the rate constants in the 33 Å (hexadecane) membranes are some 3-fold less than in the 48 Å (decane) membranes. Since both the opening and closing processes are similarly affected, the probabilities of occurrence of the various conducting levels are unchanged.

The addition of cholesterol to membranes formed with hexadecane has little influence on their thickness [12]. The rate constants, as shown in Table IV, are larger than in the absence of the cholesterol, but only slightly so. In glycerol monooleate-decane membranes, by contrast, cholesterol has a much larger effect (Table VI) in that both opening and closing rate constants are smaller by a substantial amount. The thickness of the hydrocarbon region of these membranes is 35 Å, compared with 48 Å before the addition of cholesterol, and this decrease may account for part of the change in the rate constants. Further evidence supporting the importance of bilayer

TABLE IV

THE EFFECT OF CHOLESTEROL ON THE STABILITY OF ALAMETHICIN-CONDUCTING STATES

System: Glycerol monooleate (6 mM)-(cholesterol, 14.8 mM)-hexadecane/2 M KCl, −220 mV.

(A) Fraction of the time (T_i/T_{total}) spent in each conducting state during the period of conduction

	State							
	1	2	3	4	5	6	7	8
With cholesterol	0.005	0.039	0.13	0.39	0.33	0.095	0.018	0.001
Without cholesterol	0.006	0.020	0.14	0.38	0.34	0.098	0.015	0.002

(B) Rate constants for the opening (↑) and closing (↓) of channels (s^{-1})

	$k_{1\uparrow}$	$k_{2\uparrow}$	$k_{3\uparrow}$	$k_{4\uparrow}$	$k_{5\uparrow}$	$k_{6\uparrow}$	$k_{7\uparrow}$
With cholesterol	6400	3000	2500	620	390	400	350
Without cholesterol	2700	2000	1200	480	280	280	260
	$k_{2\downarrow}$	$k_{3\downarrow}$	$k_{4\downarrow}$	$k_{5\downarrow}$	$k_{6\downarrow}$	$k_{7\downarrow}$	$k_{8\downarrow}$
With cholesterol	830	700	840	700	1300	1900	7100
Without cholesterol	800	300	420	540	970	1900	2000

TABLE V

THE EFFECT OF SOLVENT HYDROCARBON CHAIN LENGTH ON TRANSITIONS BETWEEN OLIGOMER-CONDUCTING STATES

(A) Fraction of the time (T_i/T_{total}) spent in each conducting state; system: glycerol monooleate (6 mM)-decane/2 M KCl, 223 mV

	State						
	1	2	3	4	5	6	7
T_i/T_{total}	0.01	0.05	0.26	0.39	0.22	0.07	0.02

(B) Rate constant ratios (for hexadecane data see Table IV)

	State						
	1	2	3	4	5	6	7
$k_\uparrow$ glycerol monooleate-decane/ $k_\uparrow$ glycerol monooleate-hexadecane	2.9	2.4	1.3	2.0	3.6	4.3	–
$k_\downarrow$ glycerol monooleate decane/ $k_\downarrow$ glycerol monooleate hexadecane	–	2.2	2.9	2.4	3.1	3.3	3.1

thickness was obtained from experiments using glycerol monooleate-hexadecyl bromide membranes where the hydrocarbon region is only approx. 25 Å [13], and for which the rate constants were smaller than in either the glycerol monooleate-hexadecane or the glycerol monooleate-cholesterol-decane systems.

In membranes formed from phospholipids, the rate constants tended to be much smaller than in those formed from monoglycerides (cf. Tables I and V). Within the phospholipids (e.g. phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine), however, the variations in the rate constants were relatively small. In Table VII is listed the whole range of systems examined in order of increasing rate constants. The actual data on which this table is based are not given because they were obtained under slightly different conditions of alamethicin concentration and applied potential and are therefore not strictly comparable. It is nevertheless unlikely that the order is incorrect and, as a guide, there are approximately two orders of magnitude between the top and bottom systems.

TABLE VI

THE EFFECT OF CHOLESTEROL ON THE RATE CONSTANTS FOR TRANSITIONS BETWEEN CONDUCTING STATES

Systems: glycerol monooleate-(cholesterol, 14.8 mM)-decane/2 M KCl, −223 mV.

	State					
	1	2	3	4	5	6
$k_\uparrow$ cholesterol/$k_\uparrow$ no cholesterol	0.16	0.093	0.14	0.087	0.21	–
$k_\downarrow$ cholesterol/$k_\downarrow$ no cholesterol	–	0.16	0.20	0.40	0.24	0.30

TABLE VII

THE EFFECT OF LIPID COMPOSITION ON THE RATE CONSTANTS FOR TRANSITIONS BETWEEN ALAMETHICIN-CONDUCTING STATES

Membrane: (rate constants increase from top to bottom)	Hydrocarbon thickness (Å)	Double bonds per cm^2 membrane ($\times 10^{-16}$)
Bacterial phosphatidylethanolamine-decane	42	0.026
Egg phosphatidylcholine-cholesterol-hexadecane	32	–
Stearyl palmitoleyl phosphatidylcholine-cholesterol-hexadecane	(32)	–
Glycerol monooleate-hexadecyl bromide	25	$\approx$ 0.04
Egg phosphatidylethanolamine-decane	(48)	0.112
Brain phosphatidylserine-decane	50	0.078
Dioleyl-phosphatidylcholine-decane	48	0.062
Glycerol monooleate-cholesterol-decane	35	–
Glycerol monopalmitoleate-cholesterol-hexadecane	(28)	–
Glycerol monooleate-hexadecane	33	0.052
Glycerol monooleate-cholesterol-hexadecane	(30)	–
Glycerol monooleate-decane	48	0.052

The trends and values reported in this paper for the rate constants of the transitions for phospholipid membranes agree qualitatively with results obtained by Boheim [8]. A quantitative comparison is not possible owing to differences in the type of lipid used and in the temperatures and potentials at which the results were recorded.

Electrolyte, pH and surface charge effects. The rate constants do not appear to be affected by changes in the type of electrolyte used, although it is possible that in glycerol monooleate systems the presence of high concentrations of citrate and Ca^{2+} (in separate experiments) cause slight increases and decreases, respectively, in the rates of change of the states of conduction. The effect of pH is not very large. Rate constants are approximately independent of pH between 2.8 and 10 for transitions between the fourth and fifth levels but increasing the H^+ concentration tends to increase the rates at high conductance levels and the reverse occurs at lower levels. Since forward and backward rates are affected similarly, the H^+ presumably acts as a catalyst.

Membranes made from 1 : 1 glycerol monooleate/oleylamine in decane (pH 5.6) were more stable in the presence of alamethicin than were simple glycerol monooleate-decane films. Nevertheless the rate constants were identical. Similarly, glycerol monooleate-hexadecane-cholesterol membranes gave identical results with respect to the pattern of alamethicin conductance to similar membranes containing the negatively charged cardiolipin. Although it was not possible to determine the percentage of cardiolipin or oleylamine in the films, the results suggest that symmetrically placed charges on the two membrane surfaces do not have any effect on the movement of alamethicin. This conclusion is supported by the experiments with phosphatidylserine and phosphatidylethanolamine, which gave identical results at pH 7.

DISCUSSION

Surface studies have shown that alamethicin adsorbed at monoglyceride-decane/aqueous solution interfaces is mainly in the monomeric form [5]. However, as the conductivity of bilayers containing alamethicin is dependent on the ninth power of the peptide concentration [3, 5] and, as each burst of conductivity is multi-valued, it is almost certain that these bursts originate from oligomers of the peptide. As the applied potential is increased, the membrane conductance rises but shows no sign of saturating before it greatly exceeds that of the aqueous solution, and hence is no longer measurable. It seems, therefore, that under normal conditions, only a small fraction of the alamethicin in the membrane takes part in the conduction process. Since the conductance vs. voltage curve is moved about 90 mV along the voltage axis for each 10-fold increase in the surface concentration of the alamethicin (assuming the surface and bulk concentrations to be directly proportional when the latter is less than 10^{-6} M [3, 5]) and since it is normal to observe 3.5–4 decades of the linear $\log_{10}$ (conductance) versus applied potential plot, it can be calculated that when one oligomer is conducting there must be at least 10^5 adsorbed alamethicin molecules.

It was pointed out in Introduction that the various levels of conductance in the oligomers could arise in two distinct ways (a) from conformational changes of the pre-formed oligomers, or (b) from variations in the number of molecules in the oligomer. As the forces involved in the formation of conducting oligomers (i.e. peptide-peptide interactions and peptide-membrane field interactions) are mutually orthogonal, it is reasonable to formulate the mechanisms in terms of two sequential processes. In so doing, non-conducting intermediates are postulated for which there is no independent evidence but which are convenient for purposes of discussion. Thus, omitting the details [5], mechanism (a) could be written

$$n\mathrm{A} \overset{K_a}{\rightleftharpoons} \mathrm{A}_n \overset{K_{a(E)}}{\rightleftharpoons} \mathrm{A}_n^*$$

where the asterisk denotes the conducting species. Only the second part of the process (equilibrium constant $K_a(E)$) would be field dependent.

Mechanism (b), on the other hand, could be written

$$n\mathrm{A(s)} \overset{K_{b(E)}}{\rightleftharpoons} n\mathrm{A} \overset{K_b}{\rightleftharpoons} \mathrm{A}_n^*$$

where A(s) are alamethicin molecules on the surface of the membrane and A are molecules which have responded to the electric field. It is assumed that these molecules would not conduct appreciably since bursts of current of only one conducting level are very rarely seen. This is particularly obvious for "negative" applied potentials*. A_n^* are the conducting species and n can have at least nine integral values corresponding to the nine conducting levels which may be observed [5]. Thus,

$$\frac{[\mathrm{A}_n^*]}{[\mathrm{A}]^n} = (K_b)_n \qquad n = 1, \ldots, 9.$$

* It has been argued in ref. 5 that, contrary to earlier suggestions, channel formation for 'negative' applied potentials does not first involve diffusion of alamethicin to the far side of the membrane, but rather an inversion of the polypeptide through the interaction of its dipole with the reversed field.

One possibility of distinguishing between mechanisms (a) and (b) is to examine the field dependence of the conduction level probability during the functioning of (a) single oligomer (in the case of a) or of a sequence of oligomers (as required by b). In a, the field dependence observed in the conduction of the whole membrane arises principally from the formation of more conducting oligomers, and although the field could also have some effect on the level probability within an oligomer, this is in no sense necessary and would have to be a relatively small effect. In (b) the field dependence for the whole membrane also arises from the formation of more oligomers but, since it is the concentration of monomeric alamethicin in the membrane which is directly affected by the field, there is necessarily a change in the relative concentrations of the different size oligomers. For a single burst of conduction this would be manifest as an increase in the probability of occurrence of the higher levels. In using this approach, it is obviously necessary that the voltage pulse employed to change membrane field should be sufficiently long for the concentration of alamethicin monomers in the membrane to change appreciably and hence that the oligomer which is being observed should have a life much longer than the statistical mean.

The results (Table III) are sufficient to show the effect of the field change on the equilibrium between levels two and three, and between three and four. As can be seen, there is an increase in probability of the higher level in the first instance, but a decrease in the second. Assuming that the response to the field of the monomeric alamethicin is through a dipole [5] and assuming this quantity, divided by its length, to be such that it would account for the observed I vs. V curves at high levels of conductance, it can be calculated that $k_{4\uparrow}/k_{3\downarrow}$ as well as $k_{3\uparrow}/k_{2\downarrow}$ should increase approx. $\times 1.3$ for a 10 mV increase in applied potential. These data suggest, therefore, that mechanism (b) is incorrect. They could also be taken to indicate that in mechanism (a) there is some field dependence of the level probability. In the experiment in which the field is varied without keeping constant the alamethicin concentration (Fig. 6 and Table II) the result is similar to that just discussed, as would be expected if mechanism (a) operated.

There is little else in the experimental data which helps to distinguish between mechanisms (a) and (b). If (b) were to be correct (in spite of the above evidence) the rate-limiting step in the establishment of the conduction would not be the diffusion of the monomers through the lipid molecules to the conducting sites. This may be argued from the fact that, although the rate constants for the increasing conduction steps in an isolated burst of current are dependent on the type of membrane, they are not equal, in any given membrane, for the various levels. Theoretical estimates of diffusion rates in the plane of the membrane are not helpful except insomuch as they do not rule out (b) as a possible mechanism.

The thermodynamic studies (Table I) have shown how, for model (a), the standard free energy, enthalpy and entropy differences between the levels vary. The zero enthalpy difference between levels, as well as the corresponding very low activation energies (approx. 1.2 kcal/mol) suggest strongly that the opening and closing of the channels as the levels change, does not involve any substantial molecular reorganization. In mechanism (a), the entry into, and exit from the membrane of the pre-formed oligomer would control the rates of increase and decrease of the macroscopic conductance. With a very low ratio of conducting to non-conducting oligomers, the rate constants for this process would be approximately first order, as observed, and would

account for the non-sigmoidal rise in the conductance following a voltage step. Alamethicin is extremely insoluble in hydrocarbons and, because the molecule contains two prolines, internal hydrogen bond formation would be restricted. It is possible, therefore, that the surface-formed oligomers are held together by hydrogen bonds and, for this reason, may enter the membrane relatively easily compared to the monomers, which have unsatisfied hydrogen bonds.

It has been pointed out that, while the equilibrium probabilities of the conducting levels of an oligomer are scarcely affected by the membrane composition, the rate constants for transitions between the levels are very sensitive to this composition. From Table V it seems that the thickness of the hydrocarbon part of the membrane may be of some importance in this respect, but the increase of approx. $\times 3$ in the rate constants as the membrane thickness increases from 33 to 48 Å is small compared with the two orders of magnitude in the total variation. The addition of cholesterol to many black film systems reduces the thickness and there seems to be a concomitant reduction in the rate constants (Table VII). For films in which cholesterol does not affect the thickness, however, its effects seem to be minimal (Table IV). The lipid polar groups may be of some significance in that the slower rate constants are found in phospholipid membranes, but the correlation is not complete. In fact, no single quantitatively known property, or any likely combination of these properties, seem to offer any explanation. As it is the rate constants rather than the equilibrium constants which are affected, the membrane fluidity is an obvious property to consider but, in the absence of any quantitative data, little further can be said. The double bond content of the membranes, which is often thought to be an indication of fluidity, is listed in Table VII but, as can be seen, there is, even here, no clear correlation.

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