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Analysis of the Multi-pore System of Alamethicin in a Lipid Membrane

I. Voltage-Jump Current-Relaxation Measurements

Günther Boheim and Hans-Albert Kolb

Fachbereich Biologie, Universität Konstanz, D-7750 Konstanz, Germany

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Summary. The electrical properties of an alamethicin multi-pore system have been studied by voltage-jump current-relaxation experiments (this paper) and by autocorrelation and spectral analysis (following paper). With these methods a slow time constant and a fast time constant were observed which differ by about one to three orders of magnitude depending on the experimental conditions. Steady-state current and time constants were analyzed as functions of voltage, alamethicin concentration and temperature. Within experimental error the data obtained with these different methods are in good agreement. The experimentally measured relation between the voltage and alamethicin concentration dependence of the slow relaxation time fits into a model of an alamethicin pore which adopts consecutive pore states and which decays only from the lowest state. It indicates that the uptake of one alamethicin molecule by the existing pore and, in formal equivalence, the transfer of about one positive elementary charge across the membrane are associated with the transition from a given pore conductance state to the next higher state. From the voltage and alamethicin concentration dependence of the pore formation rate evidence shows that a hexameric preaggregate exists at the membrane interface out of which two to three molecules are simultaneously inserted into the membrane to form the pore nucleus. The effects of different voltage pretreatment on the experimentally determined parameters have been investigated and are discussed in detail.

Alamethicin has been shown by Mueller and Rudin (1968), Baumann and Mueller (1974) and Mueller (1976*a*, *b*) to be a model substance which induces excitability phenomena in artificial lipid membranes. A way to elucidate the underlying molecular mechanism of alamethicin action has been opened through single channel investigations by Gordon and Haydon (1972), Haydon, Hladky and Gordon (1972), Eisenberg, Hall and Mead (1973), Boheim (1974), Gordon (1974), Gordon and Haydon (1975), Hall (1975), Gordon and Haydon (1976), Mueller (1976*a*), and Gisin, Kobayashi and Hall (1977). It was shown that alamethicin forms pores

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which open and close in a voltage-dependent way. Each of the pores fluctuates between different states. These pore states exhibit a peculiar conductance sequence which does not originate from integral multiples of a unit conductance step and they occur in consecutive order. Whereas the mean life-times of the pore states lie in the millisecond (ms)-range, the pore may persist up to several seconds.

The molecular picture of the pore structure is controversial: Gordon and Haydon (1975) proposed an aggregate of parallel pores which all show similar properties. The nonintegral and nonlinear conductance properties are thought to result from the central hole which is created by the bunch of surrounding pores and from interactions between them. On the other hand, Baumann and Mueller (1974) and Boheim (1974) proposed the "barrel stave model". They assume that the pore consists of a pore nucleus which grows in diameter by uptake of single alamethicin molecules and disappears by release of these subunits. Hall (1975) pointed out that the pore might be of a fixed number of molecules. Alamethicin molecules are supposed to form oligomers (micelles) at the membrane interphase and the conducting pore with its different states is created by configurational changes of the oligomer. Eisenberg *et al.* (1973) and Mueller (1976*a*) pointed out that some kind of preaggregation has to occur before the active pore is induced by the applied voltage.

Concerning the voltage dependence of single-pore state distributions different results were reported. It is found that the most probable pore state changes by one level for every 40–50 mV (Boheim, 1974), 60 mV (Mueller, 1976*a*), 100 mV (Gordon & Haydon, 1975, 1976), whereas nearly no voltage dependence was found by Eisenberg *et al.* (1973) and Hall (1975). This discrepancy may be a consequence of different experimental procedures. Whereas Boheim (1974) made his analysis at the different voltages at single long-living pores (mean life-time >1 min at low temperature), Eisenberg *et al.* (1973), Hall (1975) and Gordon and Haydon (1975, 1976) averaged over many relatively short living pores (at room temperature). In order to obtain clear single-pulse records over a range of potential it was necessary to vary concomitantly the alamethicin concentration (Gordon and Haydon, 1976). The evaluation of the voltage-dependence of pore state distributions as reported by Boheim (1974) led to the supposition that, in formal equivalence, about one positive elementary charge is transferred across the membrane during each transition from a distinct to the next higher pore conductance state. For the following we want to emphasize the observation that a pore preferentially first adopts its lowest conductance state and also preferentially disappears

from that state (Eisenberg *et al.*, 1973; Boheim, 1974; Gordon & Haydon, 1976).

Multi-pore experiments with alamethicin have been published by Mueller and Rudin (1968), Cherry, Chapman and Graham (1972), Eisenberg *et al.* (1973), Boheim (1975) and Roy (1975). Besides a weakly voltage-dependent ('voltage-independent') part of the alamethicin-induced conductance which is observed with most lipids, a voltage-dependent part with a very steep characteristic can be seen. Two different relaxation processes were observed with voltage-jump current-relaxation (voltage-clamp) measurements (Baumann & Mueller, 1974). The slow relaxation seems to originate from a single first-order process (Eisenberg *et al.*, 1973; Boheim, 1975; Roy, 1975). The corresponding relaxation time τ_s increases with voltage. Alamethicin concentration dependence measurements have been reported by Eisenberg *et al.* (1973) and Roy (1975) for the steady-state voltage-dependent (λ_∞) and voltage-independent (λ_Φ) conductances. They found a 9^{th}–10^{th} power dependence on alamethicin concentration for λ_∞ and a 2^{nd}–3^{rd} power dependence for λ_Φ. In a previous communication (Boheim, 1975) we reported about the alamethicin concentration dependence of the slow relaxation time τ_s. With increasing alamethicin concentration an increase in τ_s is observed. The dependence of λ_∞ on salt concentration has been measured by Eisenberg *et al.* (1973) at room temperature. They found a 4^{th} power dependence. Gordon and Haydon (1975) demonstrated on the basis of adsorption experiments of alamethicin to a glyceryl monooleate-decane/NaCl solution interphase that the amount of adsorbed alamethicin molecules is approximately dependent on the square root of the NaCl concentration in the range 0.2 to 1.0 M at 20° C. Thus, the ionic strength effect of alamethicin adsorption to the membrane seems to account for the entire salt concentration dependence of λ_∞. The original experiments of Mueller and Rudin (1968) were done at 35 °C. They reported a 6^{th} power dependence of λ_∞ on alamethicin concentration. The difference to the earlier mentioned value may result from the different temperatures.

Alamethicin is thought to be a linear polypeptide (Jung, Dubischar & Leibfritz, 1975; Martin & Williams, 1975, 1976). The N-terminus seems to be a N-acetylated methylalanine and the C-terminus of the R_F30 component the free α-carboxyl group of a glutamine. The γ-carboxyl group of the glutamic acid which precedes the glutamine is blocked by a phenylalaninol thus forming an end-branched polypeptide. In organic solution about 35–40 % of the molecule seems to be in α-helical configuration starting from the N-terminal end. The rest seems to be quite flexible

and could possibly adopt a β-bend structure (Boheim, Janko, Leibfritz, Ooka, König & Jung, 1976).

Another antibiotic, suzukacillin, which is produced by a different strain of Trichoderma viride (Ooka, Shimojima, Akimoto, Takeda, Senoh & Abe, 1966; Ooka & Takeda, 1972; Jung, König, Leibfritz, Ooka, Janko & Boheim, 1976) has been demonstrated to exhibit membrane modifying effects similar to alamethicin (Boheim *et al.*, 1976). In the case of the suzukacillin multi-pore system two relaxation processes have been observed, too. The fast one which occurs in the ms-range might be a sum of different first-order relaxations of comparable time constants. It seems to reflect the ensemble of pore state transitions. On the other hand, the slow relaxation process again can be described by a single first-order reaction. Under consideration of the fact that a pore only forms and disappears by adopting its lowest conductance state, the slow reaction has been interpreted as the pore formation and decay process.

In this paper we report on a study of the alamethicin multi-pore system at different voltages, alamethicin concentrations and temperatures. The experiments were done mainly under two differently defined conditions of membrane pretreatment. Emphasis has been put on the determination of a relation between the voltage and alamethicin concentration dependence of the slow relaxation time. This relation allows the possibility of estimating the number of alamethicin molecules which are engaged in a single change of state of the fluctuating pore.

Different experimental methods have been applied to get information on the system: the voltage-jump current-relaxation technique (Ketterer, Neumcke & Läuger, 1971; Stark, Ketterer, Benz & Läuger, 1971; Bamberg & Läuger, 1973; Boheim *et al.*, 1976) which is presented in this paper and the autocorrelation and spectral analysis (Zingsheim & Neher, 1974; Kolb, Läuger & Bamberg, 1975; Moore & Neher, 1976) presented in the following paper (Kolb & Boheim, 1977).

Materials and Methods

Black lipid membranes were formed from L-α-dioleoyl phosphatidylcholine (di-(18:1)-lecithin) synthesized by *K.* Janko (Benz, Stark, Janko & Läuger, 1973). Membrane-forming solutions were made of 1% (w/v) lipid in *n*-decane.

Our experiments were made with the pure R_F 30 fraction of alamethicin checked by thin-layer chromatography in chloroform, methanol, water 65:24:4 (v/v/v). It was purchased from Microbiological Research Establishment, Porton Down, Salisbury. Alamethicin was added from a stock solution of 10^{-4} g/ml in ethanol, water 1:9 (v/v) in amounts of 10–75 µl to 10 ml

aqueous solution. The antibiotic was present in identical amounts in both aqueous compartments. Alamethicin was always added prior to membrane formation.

KCl salt solutions were 1 M, if not otherwise stated, and unbuffered (pH ~ 5.5). KCl was p.A. grade from Merck.

The measuring assembly has been described elsewhere (Boheim, 1974). Measurements were made with Teflon cells containing a hole of 1 mm diameter in multi-pore experiments and a hole of about 0.1 mm diameter in single-pore experiments, respectively. The area of the black membrane was determined with a calibrated scale in the ocular of a microscope. Aqueous solutions were stirred by Teflon-coated steel bars. Stirring was stopped after 15 min and time allowed for the system to reach a steady-state. Data were taken at least 60 min after the membrane became black.

The experimental curves were recorded by photographing from a storage oscilloscope (Tektronix, Type 549) and/or by making tape records (Telefunken, Type MAS 54; frequency response from dc to 10 kHz) and playing it back with an enlarged scale in time and amplitude.

Two Ag/AgCl electrodes were used to transmit current through the system under voltage-clamp conditions. There was no difference to the results when current and voltage were measured separately with four Ag/AgCl electrodes. Voltage steps were imposed across the membrane by a square pulse function generator (Electronic Division, University of Konstanz) with a response time in the nanosecond (ns)-range.

The experimental system was symmetric with respect to salt and alamethicin concentrations. In some experiments a slight asymmetry in the current-voltage characteristic appeared, whereby the higher conductances were observed on the side from which the membrane had been painted. Voltage will be designated positive if the more positive potential is applied on this side. Current direction is defined as positive for cation transfer from this side to the opposite one. Temperature was measured by a Pt resistance thermometer inserted into one aqueous compartment. The values were accurate within ± 1 °C.

Results

The behavior of the alamethicin system depends to a large extent on its pretreatment with respect to membrane voltage and temperature. This is caused by the occurrence of very slow processes which come off in the minute- to hour-range and which cannot be unambiguously interpreted on the basis of molecular events. Because we want to investigate processes which occur in the millisecond to second range, this difficulty should be eliminated by measuring under well-defined pretreatment conditions. Consequently we have carried out our experiments under reproducible steady-state conditions. In the following we give the definition of three voltage-pretreatment and two temperature-pretreatment conditions.

Voltage-Pretreatment A (Zero Voltage-Pretreatment)

The system was stirred for 15 min after the membrane became black. The membrane was maintained at zero voltage. The experiments were started after either additional 45 min (at 25 °C) or 105 min (at 12 °C and lower temperatures). The results were quite reproducible if the voltages were applied only for very few seconds and if small conductances $\lambda_\infty < 10\ \mu S\ cm^{-2}$

were induced under the given conditions. Otherwise a steady drift of λ_∞ to higher values was observed at constant voltage.

Voltage-Pretreatment B (Low λ_Φ-Pretreatment)

The system was stirred for 15 min after the membrane became black. During additional 45 min (at 25 °C) the membrane was maintained at zero voltage. Then a few voltage-jumps to the highest voltage used were done until a steady-state in conductance λ_∞ ($\lambda_\infty > 1$ mS cm^{-2}) and a reproducible slow relaxation process was reached. In order to obtain a steady-state the duration of the square pulses and the pause in between had to be kept constant. Subsequently, a series of positive voltage-pulses starting from 0 mV with decreasing amplitude were carried out. We recorded three relaxation curves at each voltage. The length of the voltage pulses was approximately 5 times the slow relaxation time at the highest voltage used, whereas the pause period between the pulses lasted about 50 times this time constant. After having recorded the whole series we started for another one again at the highest voltage used. Under the above given pretreatment conditions the weakly voltage-dependent conductance λ_Φ usually remained constant and below 8 μS cm^{-2} during the time of the experiments.

Voltage-Pretreatment C (High λ_Φ-Pretreatment)

The system was stirred for 15 min after the membrane became black. A distinct voltage within the voltage-dependent range was applied and the system was held at a conductance λ_∞ between 0.1 and 1 mS cm^{-2} by proper adjustment of the voltage during a time period of up to 2 hr. During this time the weakly voltage-dependent conductance λ_Φ increased up to values of 100 μS cm^{-2}. Then the largest voltage used was applied and after about an additional 15 min a steady-state was reached. From this voltage a jump of 10 mV to lower values and back to the initial voltage was done while the current-relaxations were recorded. The length of the pulse interval was about 5 times the slow relaxation time at the highest voltage used and the pause period lasted nearly 50 times this time constant. After having recorded three relaxation curves the preconditioning voltage was reduced in steps of 5 mV. In each case we again waited 15 min for a steady-state before the relaxation curves were obtained by the application of 10 mV square pulses to lower voltages. A second series of data was taken in the same manner by starting again at the highest voltage used.

Temperature-Pretreatment A

The membrane was always formed at 25 °C and then relaxation curves were recorded following the low λ_Φ- or high λ_Φ-pretreatment, respectively. Thereafter the temperature was lowered to 18 °C. 1.5 hr later the measurements were done in the same sequence of decreasing voltage amplitudes as described above. The same procedure was carried out at 11 °C and under conditions of low λ_Φ-pretreatment at 4 °C. On the other hand, under conditions of high λ_Φ-pretreatment the temperature was raised again to 18, 25 and 32 °C. Membranes with which the measurements were done at all these temperatures lasted 12 to 20 hr. The experimental values of measurements on membranes after 2 and 12 hr of incubation did not differ significantly. If a membrane broke during this time, the experimental series had to be started again with a new membrane at 25 °C. After unsuccessful formation of three to four membranes it was necessary to clean the set-up and to exchange the aqueous solutions.

Temperature-Pretreatment B

The membrane was formed at the temperature at which the measurements were carried out. After the experiments were done, the membrane was broken. With a cleaned set-up and exchanged aqueous solution a new membrane was formed at the next different temperature.

Single-Pore Experiments

For comparison of multi-pore data with results obtained on the single-pore level the temperature dependence of the conductances Λ_ν and mean life-times τ_ν of single pore states ν, $\nu=0, 1, 2, 3, \ldots$ has been measured. $\nu=0$ means the nonconducting and $\nu=1$ the first conducting pore state (Boheim, 1974). Fig. 1*a* shows a plot of $\log \Lambda_\nu$ versus $1/T$ for the three most probable pore states $\nu=3, 4, 5$. The activation energy is estimated to be 3.8–4.2 kcal/mol in each case.

The conductance values have been obtained at 100 mV. The current-voltage characteristic of any single-pore state shows a slight bend to the current axis, but up to 150 mV no divergent behavior of the first conductance state from that of the others could be observed. In lecithin-decane membranes of the Mueller-Rudin type variations in Λ_ν up to a factor of 2 can be seen. Whereas the ratios of neighboring pore state conductances do not change appreciably, the absolute values may differ from one pore to the next. Even during a single-pore fluctuation the pore state conductances may change abruptly by a factor of 1.2, for instance. In general, lower conductance sets are observed just after the membrane becomes black. The Λ_ν values increase stepwise with time, whereby sometimes a transient decrease may occur. The largest conductances are measured with nearly solvent-free membranes of the Montal-Mueller type (Montal & Mueller, 1972; Benz, Fröhlich, Läuger & Montal, 1975). This increase may be a consequence of the slow change in thickness of a lecithin-decane bilayer (Bamberg & Benz, 1976) and/or of an inhomogeneous distribution of decane in the film possibly in form of microlenses (Henn, Decker, Greenawalt & Thompson, 1967).

In view of these observations the single-pore measurements were started 2 hours after the membrane became black under conditions of zero voltage-pretreatment. The results were obtained with the same membrane by lowering the temperature from $+12$ to -1 °C (temperature-pretreatment *A*).

In addition, the logarithm of the mean life-times τ_ν of the pore states $\nu=3, 4, 5$ have been plotted in Fig. 1*b* as a function of $1/T$. For each ex-

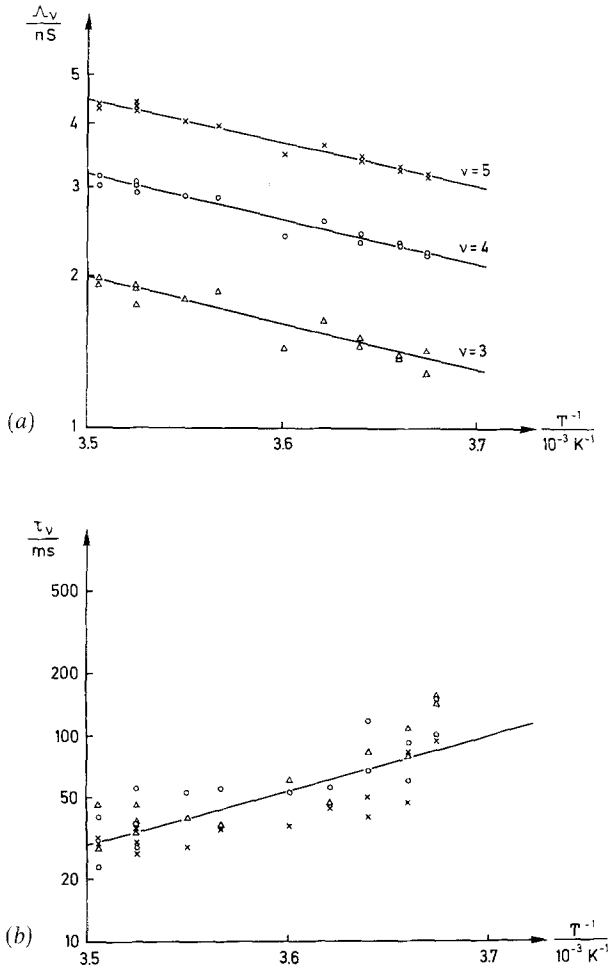


Fig. 1. Temperature dependence of (*a*) conductances Λ_v and (*b*) mean life-times τ_v of the most probable pore states $v=3$ (△), $v=4$ (○) and $v=5$ (×). The activation energies are calculated to be about (*a*) 4 kcal/mol and (*b*) 12 kcal/mol. For details *see text*. Membrane solution: 1 % di-(18 : 1)-lecithin in decane. Salt solution: 1 M KCl, unbuffered. Antibiotic concentration: nominally 10^{-7} g/ml alamethicin R_F30 on both sides. Applied voltage: 100 mV

perimental point at least 30 events were evaluated. As a consequence of the dependence of τ_v on the mean pore state $\bar{v}$ (Boheim, 1974) and because $\bar{v}$ changed to some extent from one record to the other, the reproducibility for a given τ_v lies within a factor of 2 to 3. In order to estimate the activation energy we averaged over all measured τ_v values. The activation energy is found to be about 12 kcal/mol.

Current-Relaxation Experiments

An important question which was tested first is the reproducibility of the alamethicin-induced multi-pore phenomena in lecithin bilayer membranes. At present it is not possible to measure the actual concentration of alamethicin adsorbed at the membrane-solution interface. Furthermore, we have no indication of the distribution of these molecules between conformations of different α-helical content, which have been found in solvents of varying polarity (Jung *et al.*, 1975). Comparable to the situation with suzukacillin (Boheim *et al.*, 1976), the activation energy of different molecular conformations for insertion into the membrane might be different.

1. *Experiments under conditions of zero voltage-pretreatment* (A). Under identical conditions at 25 °C we recorded several series of current-voltage characteristics using a triangle function generator to impose the voltage across the membrane. About 1 hr after the membrane became black two to three current-voltage curves were recorded up to conductances of 10 mS cm^{-2} with a pulse period of 100 sec, i.e., a voltage sweep rate of 2.4 to 3.0 mV sec^{-1}. In the conductance range of 10 μS cm^{-2} to 10 mS cm^{-2} the characteristics can be fitted quite well by an exponential function of voltage. In terms of

$$\lambda_\infty \propto \exp\{\alpha[\lambda_\infty] \cdot FV/RT\} \tag{1}$$

with F, Faraday constant; R, gas constant; T, absolute temperature; V, applied voltage; a mean value of $\alpha[\lambda_\infty] = 6.9 \pm 0.3$ is obtained from 12 series of experiments in 1 M KCl.[1] The hysteresis was less than 1 mV in the voltage-dependent part of the current voltage curve.

The variability of a characteristic voltage V_c (Eisenberg *et al.*, 1973) was determined. We define V_c as the voltage at which the exponentially extrapolated voltage-dependent conductance reaches 1 μS cm^{-2}. Under the experimental conditions of our set-up (membrane area $\sim 7 \times 10^{-3}$ cm^2) this would approximately correspond to the mean conductance of 2–3 pores in the third to fourth conductance state. We obtained $V_c = 30.7 \pm 4.4$ mV at 1 M KCl, 2.5×10^{-7} g/ml alamethicin $R_F 30$ on both sides and 25 °C. An equivalent characterization of the system is given by the

1 The indices λ_∞, λ_s, τ_s, τ_f, etc. of the parameters α, δ and ε have been included in square brackets to obtain a clearer presentation. Functional dependences are indicated by round brackets as usual.

voltage V_{100} needed to induce a conductance of 100 μS cm^{-2}. The latter definition might be more appropriate in the case of multi-pore systems. A value of $V_{100} = 47.7 \pm 4.5$ mV was obtained. Within a series of experiments with a given membrane, V_c and V_{100} did not change by more than ± 1.5 mV. The standard deviation in V_{100} of 4.5 mV in the strongly voltage-dependent conductance range corresponds to a much larger deviation in the conductance values. We obtained $\lambda_\infty = \exp\{4.6 \pm 1.1\}$ μS cm^{-2}, i.e., $33 < \lambda_\infty < 300$ μS cm^{-2} at the average value of $V_{100} = 47.7$ mV.

2. *Experiments under conditions of low λ_Φ-pretreatment (B) and temperature-pretreatment A.* A voltage which lies within the voltage-dependent conductance range and which is applied for several seconds across the membrane induces a drift of the membrane conductance. This effect is to its greater part not reversible. The weakly voltage-dependent ('voltage-independent') conductance λ_Φ of the alamethicin-modified membrane appears with a time constant of hours if the membrane is maintained at zero voltage. This waiting time can be reduced to minutes by application of voltages which induce large currents. On the other hand, in the presence of large currents the membrane tends to break. In order to restrict the variability in λ_Φ in this first series of relaxation experiments, therefore, membranes were chosen which showed λ_Φ values between 1 and 8 μS cm^{-2} (except for the case of 7.5×10^{-7} g/ml alamethicin). Consequently, the experiments were done rather in a steady state than in an equilibrium state.

The time course of the current-relaxation after a voltage-jump from 0 mV (initial voltage) to 54 mV (final voltage) at 1 M KCl, 2.5×10^{-7} g/ml alamethicin and 11 °C is represented in Fig. 2*a*. If the conductance at the initial voltage is about 1–8 μS cm^{-2}, only one relaxation process of first order is visible. For a check of the accuracy of the single exponential fitting, the half-logarithmic plot of $(I_\infty - I)$ versus t has been drawn in Fig. 2*b*. The reproducibility of the λ_∞ values for different sets of experiments at a given temperature is characterized by the limits mentioned earlier for the current-voltage characteristics.

The occurrence of an S-shaped time course of the current-relaxation curves had been reported by Baumann and Mueller (1974). In the dioleoylphosphatidylcholine-decane system an S-shaped curve could be observed only for the initial two or three current-relaxations after the membrane was freshly formed (zero-voltage-pretreatment). Especially after inducing a high conductance the S-shaped time course vanished completely (Fig. 2*a*; low λ_Φ-pretreatment).

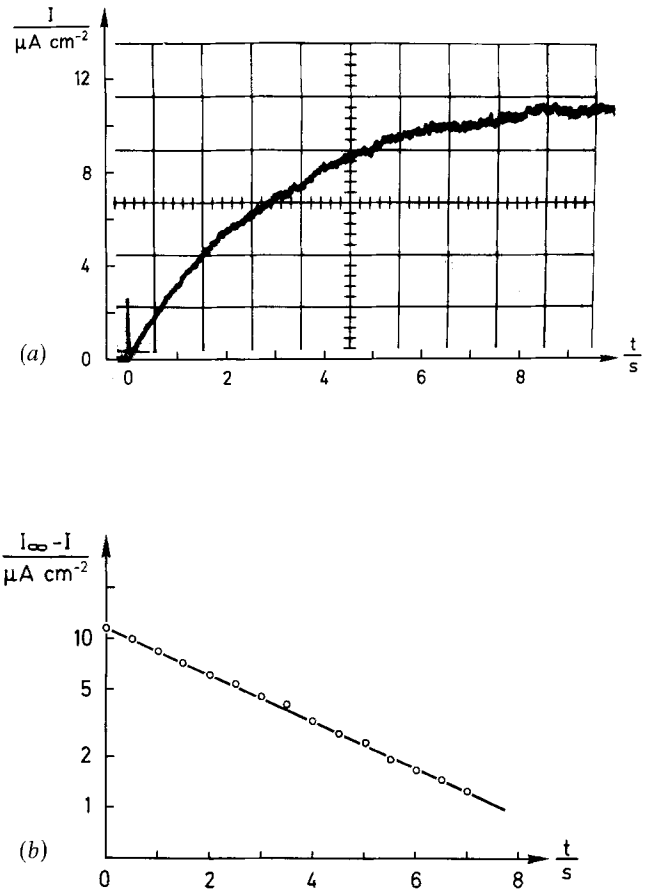
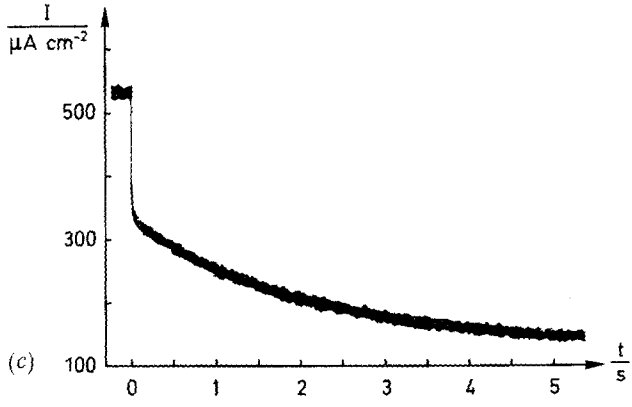


Fig. 2. Current-relaxations after a voltage-jump: (*a*) Single relaxation process of first order after a voltage-jump from 0 to 54 mV under conditions of low λ_Φ-pretreatment. (*b*) Half-logarithmic plot of the relaxation amplitude $I_\infty - I$ versus t from curve (Fig. 2*a*) for a check of the accuracy of the single exponential fitting. (*c*) Prior to the voltage-jump a high conductance has been established by pore formation at an initial voltage of 55 mV (high λ_Φ-pretreatment). After an abrupt change from 55 to 45 mV two relaxation processes are observed. The slow process is described by a single first-order differential equation. (*d*) Half-logarithmic plot of the relaxation amplitude $I - I_\infty$ versus t from curve (Fig. 2*c*). (*e*) The time scale of curve (Fig. 2*c*) has been enlarged by a factor of 50. The fast relaxation process may be fitted by a single exponential function within the limits of experimental error. (*f*) Half-logarithmic plot of the relaxation amplitude $I - I_\infty$ versus t from curve (Fig. 2*e*). o, experimental points; ×, fast relaxation process alone (after subtraction of the slow process). (g) Prior to the voltage-jump a high conductance was established at the initial voltage of 45 mV (high λ_Φ-pretreatment). After an abrupt change from 45 to 55 mV two relaxation processes are observed. The slow process corresponds to the one shown in (Fig. 2*a*). (*h*) The time scale of curve (Fig. 2*g*) has been enlarged by a factor of 200 in order to show the fast relaxation process. Membrane solution: 1% di-(18:1)-lecithin in decane. Salt solution: 1 M KCl, unbuffered. Antibiotic concentration: 2.5×10^{-7} g/ml alamethicin R_F30 in both compartments. Temperature: 11 °C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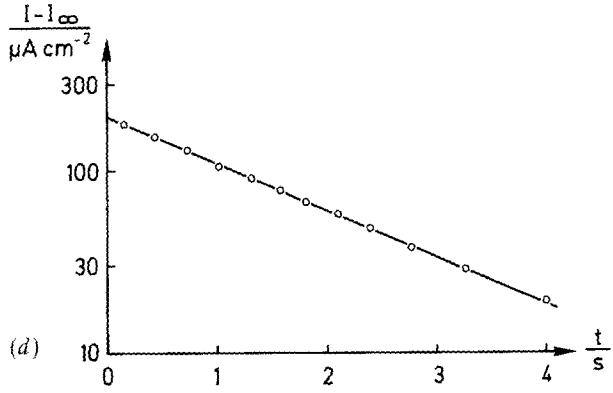
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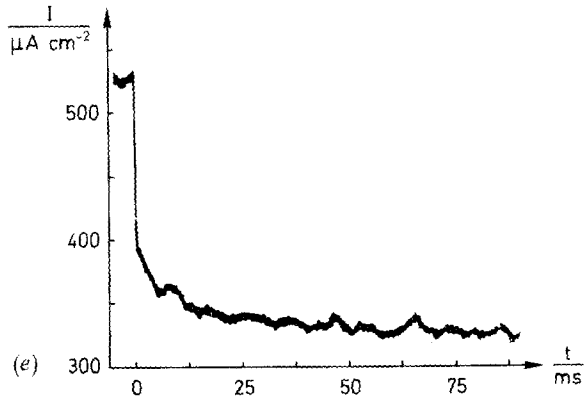
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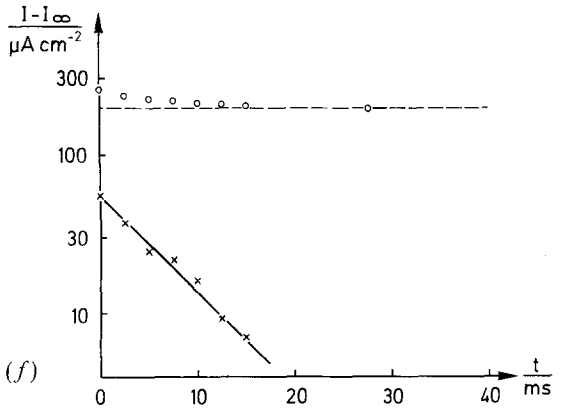
Fig. 2 *c–e*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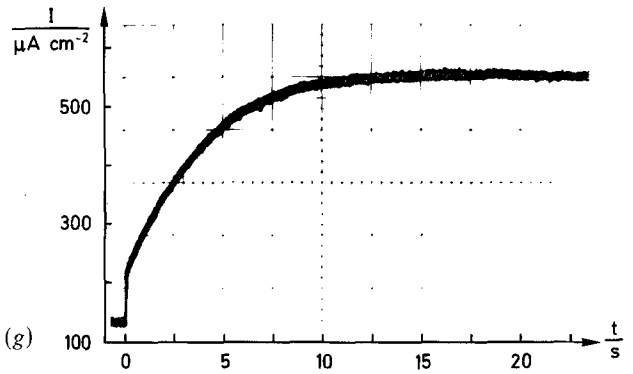
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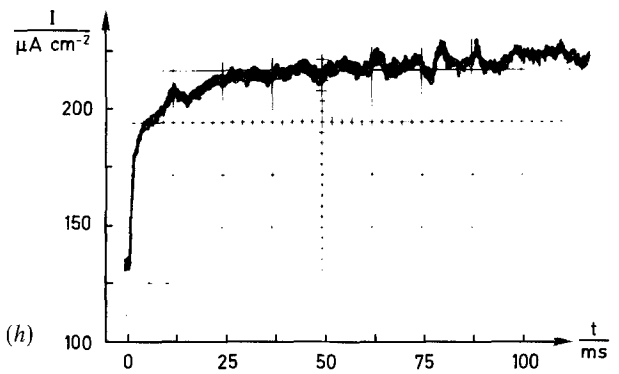
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ms

Fig. 2 *f–h*

We have carried out voltage-jump current-relaxation measurements at different temperatures. First we tested the reproducibility of the experimental results for different pretreatments. We measured the characteristic voltage V_c at 25 °C, whereby the induced conductance did not exceed 1 μS cm^{-2} appreciably (zero voltage-pretreatment). Then temperature was lowered to 4 °C (temperature-pretreatment *A*). An increase in V_c of up to 90 mV could be observed. In another experiment at 25 °C conductances $\lambda_\infty > 1$ mS cm^{-2} were induced (low λ_Φ- and high λ_Φ-pretreatment) and then the temperature reduced to 4 °C (temperature-pretreatment *A*). In this case V_c increased only by about 10 mV. In a third experiment the membrane was formed at 4 °C (temperature-pretreatment *B*) and then conductances $\lambda_\infty > 1$ mS cm^{-2} were induced (low λ_Φ- and high λ_Φ-pretreatment). By this procedure V_c decreased gradually and after many voltage-jumps (which mostly led to membrane breakage) V_c approached the value obtained with temperature-pretreatment *A*. Thus it is found that a steady-state value is reached faster (and with more experimental success) at higher temperature. At low temperature this steady state may be different from a true partition equilibrium, as there the rate of partitioning presumably becomes very small. We tested the reproducibility of the experimental data under conditions of temperature-pretreatment *A* by raising the temperature of the system to values at which measurements were already performed. Within the experimental time of 2 to 10 hr under conditions of low λ_Φ-pretreatment and up to 20 hr under conditions of high λ_Φ-pretreatment no change in the steady-state values beyond the above given limits for the current-voltage characteristics occurred.

The experimental quantities which can be determined from the first-order relaxation curve (Fig. 2*a*) are the relaxation time τ_s, the final (steady-state) conductance λ_∞ and the initial conductance λ_{s0} which is obtained by extrapolation of the slow relaxation process to $t=0$. These parameters have been determined as a function of voltage, alamethicin concentration and temperature.

Fig. 3 (*a* and *b*) shows examples for the voltage dependence of the final conductance λ_∞ at two different alamethicin concentrations and at different temperatures. In the following all voltage dependences are characterized by exponential functions with the parameter $\alpha[i]$ according to Eq. (1). The $\alpha[\lambda_\infty]$ values are listed in Table 1 and it can be seen that they are virtually independent of alamethicin concentration but increase with decreasing temperature. In contrast to λ_∞ the voltage dependence of λ_{s0}

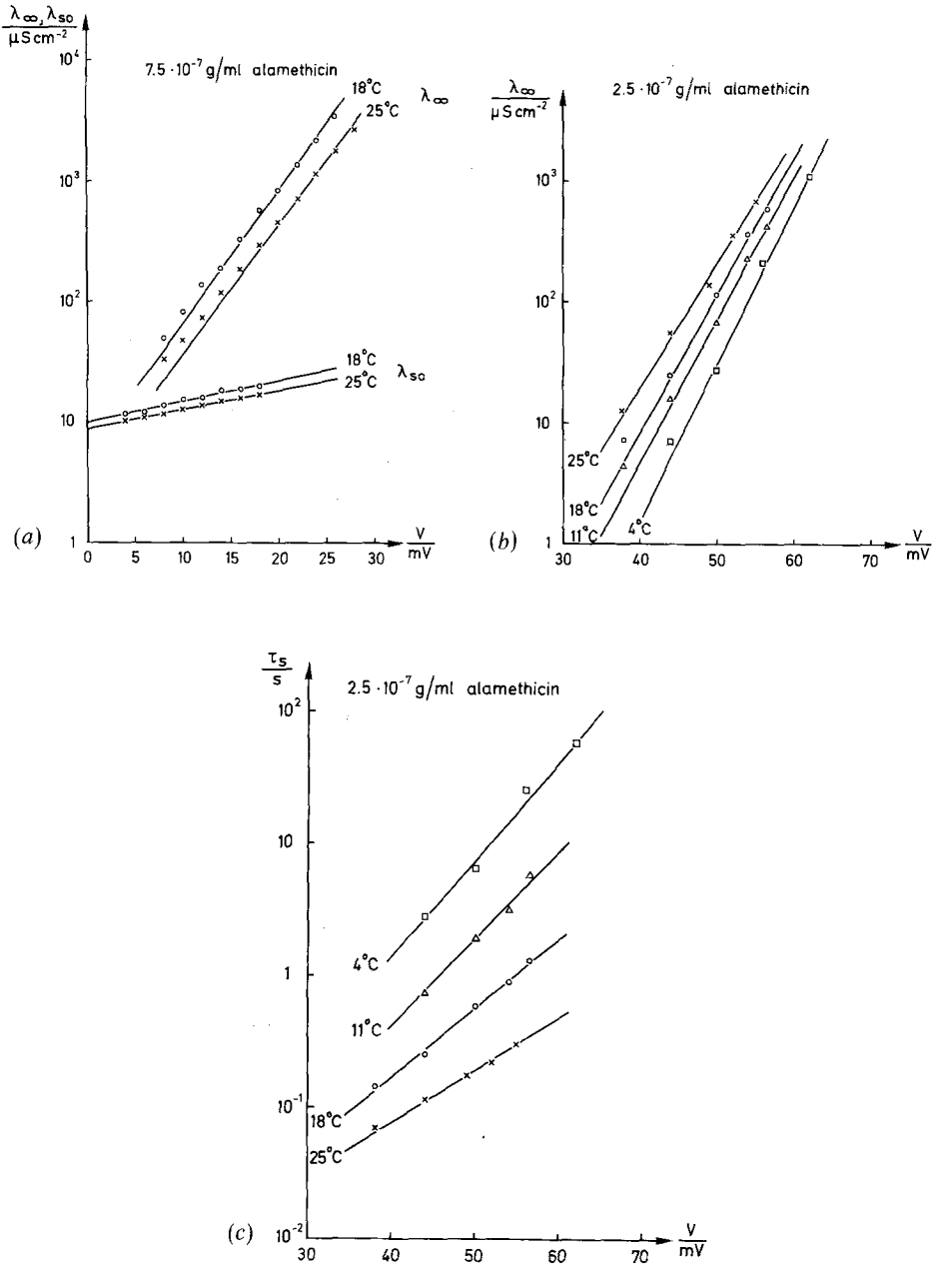

Fig. 3. (*a*) Final conductance λ_∞ and extrapolated initial conductance λ_{s0}, (*b*) final conductance λ_∞, and (*c*) slow relaxation time τ_s as functions of membrane voltage. The measurements have been carried out under conditions of low λ_Φ-pretreatment at two and four different temperatures, respectively: ×, 25 °C; ○, 18 °C; △, 11 °C; □, 4 °C. Membrane solution: 1% di-(18:1)-lecithin in decane. Salt solution: 1 M KCl, unbuffered. Antibiotic concentration: (*a*) 7.5×10^{-7} g/ml; (*b*, *c*) 2.5×10^{-7} g/ml alamethicin R_F30 on both sides

Table 1. Exponential factors $\alpha[\lambda_\infty]$, $\alpha[\tau_s]$, $\alpha[\lambda_{s0}]$, and $\alpha[\mu]$ characterizing the voltage dependence of the final conductance λ_∞, the slow relaxation time τ_s, the extrapolated initial conductance λ_{s0} and the pore formation rate μ at different alamethicin concentrations and temperatures[a]

Temp.	Exponential factor	Alamethicin concentration $C_{AL}[10^{-7}$ g/ml]						Mean value
		1.0	1.5	2.5	3.5	5.0	7.5	
25 °C	$\alpha[\lambda_\infty]$	6.46	6.36	6.09	6.25	6.53	6.29	6.3
	$\alpha[\tau_s]$	2.50	2.55	2.37	2.63	2.63	2.60	2.6
	$\alpha[\lambda_{s0}]$			0.98	0.98	0.97	0.93	0.96
	$\alpha[\mu]$							2.8
	$\lambda_{s0}(V_{100})$/ [μS cm^{-2}]	1.7	1.5	3.0	5.4	8.0	14.2	
18 °C	$\alpha[\lambda_\infty]$	6.99		6.56	6.52	6.87	6.34	6.7
	$\alpha[\tau_s]$	2.81		2.99	3.11	2.98	3.14	3.0
	$\alpha[\lambda_{s0}]$			0.93	0.80	0.85	0.97	0.89
	$\alpha[\mu]$							2.8
	$\lambda_{s0}(V_{100})$/ [μS cm^{-2}]	1.8		3.6	5.0	4.5	15.4	
11 °C	$\alpha[\lambda_\infty]$		7.51	6.66	7.04	7.08		7.1
	$\alpha[\tau_s]$		3.70	3.70	3.67	3.80		3.7
	$\alpha[\lambda_{s0}]$			0.78	0.77	0.64		0.73
	$\alpha[\mu]$							2.6
	$\lambda_{s0}(V_{100})$/ [μS cm^{-2}]		1.2	2.7	5.0	3.4		
4 °C	$\alpha[\lambda_\infty]$			7.14	6.71	7.33		7.1
	$\alpha[\tau_s]$			4.07	3.90	4.30		4.1
	$\alpha[\lambda_{s0}]$			0.73	0.88	0.67		0.76
	$\alpha[\mu]$							2.2
	$\lambda_{s0}(V_{100})$/ [μS cm^{-2}]			2.9	5.2	3.3		

[a] For definition of the $\alpha[i]$ *see text*. The values of λ_{s0} are taken at the voltage V_{100} where a conductance of $\lambda_\infty = 100$ μS cm^{-2} is reached. Data are obtained under conditions of low λ_Φ-pretreatment.
Membrane solution: 1% di-(18:1)-lecithin in decane.
Salt solution: 1 M KCl, unbuffered.

is only weak (Fig. 3*a*). $\alpha[\lambda_{s0}]$ remains nearly constant at the different alamethicin concentrations and temperatures, as can be seen from Table 1.

In order to evaluate the slope of the voltage-dependent conductance (Fig. 3*a, b*) the weakly voltage-dependent conductance λ_Φ had to be taken into consideration. As outlined later, the slow relaxation process seems to reflect the formation of new pores. This means that the conductance change with voltage of the pores already existing at zero voltage is described by $\lambda_{s0}(V)$ if the voltage-jump starts from that voltage. If then the

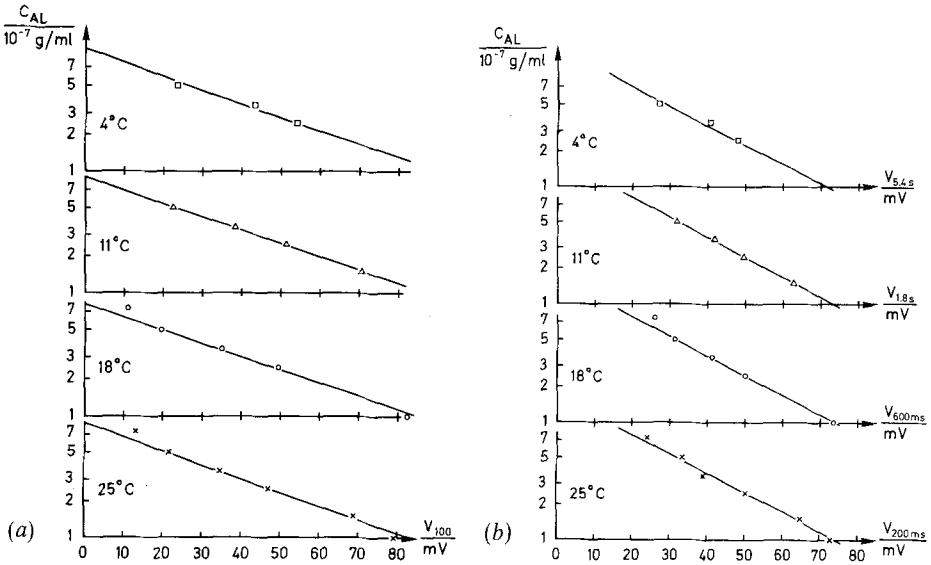

Fig. 4. (*a*) Alamethicin concentration dependence of the voltage V_{100} at which the steady-state conductance has a value of $\lambda_\infty = 100\ \mu S\ cm^{-2}$. (*b*) Alamethicin concentration dependence of the voltages $V_{200\,msec}$ at 25 °C, $V_{600\,msec}$ at 18 °C, $V_{1.8\,sec}$ at 11 °C and $V_{5.4\,sec}$ at 4 °C at which the slow relaxation time amounts $\tau_s = 200$ msec, $\tau_s = 600$ msec, $\tau_s = 1.8$ sec and $\tau_s = 5.4$ sec, respectively. The experiments were carried out under conditions of low λ_Φ-pretreatment at four different temperatures: ×, 25 °C; ○, 18 °C; △, 11 °C; □, 4 °C. Membrane solution: 1% di-(18:1)-lecithin in decane. Salt solution: 1 M KCl, unbuffered

zero voltage conductance lies within the range of the weakly voltage-dependent conductance the relation $\lambda_\Phi(V) = \lambda_{s0}(V)$ holds. Therefore, under the given experimental conditions, the solid line from which $\alpha[\lambda_\infty]$ has to be determined is given by the plot $\ln(\lambda_\infty - \lambda_{s0})$ versus t.

Fig. 3*c* gives an example for the voltage dependence of the slow relaxation time τ_s at different temperatures. The values of the $\alpha[\tau_s]$ parameter are listed in Table 1. They are independent of alamethicin concentration but increase with decreasing temperature.

The mean values of λ_{s0} at the voltage V_{100} are also given in Table 1. At least three different sets of experiments with λ_{s0} values differing from the listed value by less than a factor of 2 have been evaluated.

In order to evaluate the alamethicin concentration dependence of λ_∞ the voltage V_{100} has been plotted versus the logarithm of alamethicin concentration C_{AL} in Fig. 4*a*. Following Mueller and Rudin (1968) and Eisenberg *et al.* (1973) the alamethicin concentration dependence is described in terms of

$$\lambda_\infty \propto (C_{AL})^{\delta[\lambda_\infty]}. \qquad (2)$$

Table 2. Exponential factors $\delta[\lambda_\infty]$, $\delta[\tau_s]$, and $\delta[\mu]$ characterizing the alamethicin concentration dependence of the final conductance λ_∞, the slow relaxation time τ_s and the pore formation rate μ at different temperatures[a]

	25 °C	18 °C	11 °C	4 °C
$\delta[\lambda_\infty]$	9.4	10.7	11.5	11.7
$\delta[\tau_s]$	2.6	3.2	3.9	4.5
$\delta[\mu]$	5.8	6.5	6.6	6.2
$\frac{\alpha[\tau_s]}{\delta[\tau_s]}$	0.98	0.94	0.95	0.91

[a] For definition of the $\delta[i]$ *see text*. The (mean) $\alpha[\tau_s]$ values are taken from Table 1. Conditions are the same as for Table 1.

At constant λ_∞ and under consideration of Eq. (1) the relation

$$(C_{AL})_1^{\delta[\lambda_\infty]} \cdot \exp\left\{\alpha[\lambda_\infty] \cdot \frac{FV_1}{RT}\right\} = (C_{AL})_2^{\delta[\lambda_\infty]} \cdot \exp\left\{\alpha[\lambda_\infty] \cdot \frac{FV_2}{RT}\right\} \quad (3)$$

holds and thus $\delta[\lambda_\infty]$ is obtained from

$$\delta[\lambda_\infty] = \alpha[\lambda_\infty] \cdot \frac{V_2 - V_1}{RT/F} \cdot \frac{1}{\ln\{(C_{AL})_1/(C_{AL})_2\}}. \quad (4)$$

V_2 and V_1 are voltages at a given conductance within the exponential range of the voltage-dependent conductance. In our analysis we chose V_{100} to be this voltage.

In Table 2 $\delta[\lambda_\infty]$ values are listed which have been obtained from Fig. 4*a* with Eq. (4) using the mean $\alpha[\lambda_\infty]$ values of Table 1. The plot of $\log \lambda_\infty$ versus $\log C_{AL}$, whereby the λ_∞ values were taken at 50 mV (partly obtained by extrapolation) yields approximately the same alamethicin concentration dependence. As a consequence of the extrapolation the deviations of the experimental points from the mean straight line are more pronounced in the latter case. The listed values of $\delta[\lambda_\infty]$ are accurate within $\delta[\lambda_\infty] \pm 1$.

A corresponding evaluation has been done in Fig. 4*b* for the slow relaxation time τ_s. Due to its strong temperature dependence the following values were chosen as reference values at the different alamethicin concentrations for the experimentally observed time range: 200 msec at 25 °C; 600 msec at 18 °C; 1.8 sec at 11 °C and 5.4 sec at 4 °C. The mean difference of a factor of 3 for a temperature interval of 7 °C (Fig. 3*c* shows an example at 50 mV and 2.5×10^{-7} g/ml alamethicin) would correspond to an activation energy of 26 kcal/mol. But as a consequence of the temperature

dependence of the $\alpha[\tau_s]$-parameter, this value changes with voltage. At a voltage of about 25 to 30 mV more negative than the characteristic voltage V_c the τ_s values at different temperatures become of the same order of magnitude in the 2 to 5 ms range.

The alamethicin concentration dependence of τ_s will be characterized by a $\delta[\tau_s]$-parameter according to a relation analogous to Eq. (2). From Fig. 4*b* the $\delta[\tau_s]$ values are calculated. They are listed in Table 2 and show a pronounced increase with decreasing temperature. They are accurate within $\delta[\tau_s] \pm 0.5$.

As can be seen from Fig. 4*a* and *b* the data obtained at 7.5×10^{-7} g/ml alamethicin seem to show stronger deviations from the straight line drawn through the other experimental points. Possibly the system had not come to a corresponding steady state at the low value of zero voltage conductance of about 10 μS cm^{-2}.

A significant value for the molecular interpretation of alamethicin action is the ratio $\alpha[\tau_s]/\delta[\tau_s]$. As outlined in the Appendix from this ratio it is possible to estimate the number of alamethicin molecules which are engaged in a single change of state of the fluctuating pore. It can be seen from Table 2 that $\alpha[\tau_s]/\delta[\tau_s]$ approximates 1 in all cases.

In addition, Tables 1 and 2 show the voltage and alamethicin concentration dependence of the pore formation rate μ, which will be introduced in a later section. If μ is described according to relations analogous to Eqs. (1) and (2), $\alpha[\mu]$ and $\delta[\mu]$ are determined by:

$$\alpha[\mu] = \alpha[\lambda_\infty] - \alpha[\tau_s] - \alpha[\lambda_{s0}], \tag{5}$$

$$\delta[\mu] = \delta[\lambda_\infty] - \delta[\tau_s] - 1. \tag{6}$$

These relations follow from Eq. (11).

In order to study the salt concentration dependence of λ_∞ and τ_s a series of experiments was recorded at 0.5 M KCl. The data are listed in Table 3. Following Mueller and Rudin (1968) and Eisenberg *et al.* (1973) the salt concentration dependence of λ_∞ is described in terms of

$$\lambda_\infty \propto (C_{\text{salt}})^{\varepsilon[\lambda_\infty]}. \tag{7}$$

At constant λ_∞ and under consideration of Eq. (1) $\varepsilon[\lambda_\infty]$ is obtained from

$$\varepsilon[\lambda_\infty] = \alpha[\lambda_\infty] \cdot \frac{V_2 - V_1}{RT/F} \cdot \frac{1}{\ln\{(C_{\text{salt}})_1/(C_{\text{salt}})_2\}}. \tag{8}$$

Analogous to Eq. (4), V_2 and V_1 are voltages at a given conductance within the exponential range of the voltage-dependent conductance. We

Table 3. Exponential factors $\alpha[\lambda_\infty]$, $\alpha[\tau_s]$, $\varepsilon[\lambda_\infty]$, $\varepsilon[N_p]$ and $\varepsilon[\tau_s]$ characterizing the voltage and salt concentration dependence of the final conductance λ_∞, the pore concentration N_p and the slow relaxation time τ_s, respectively, at different temperatures[a]

	C_{salt} [M]	$\alpha[\lambda_\infty]$	V_{100} [mV]	$\alpha[\tau_s]$	V [mV]	$\lambda_{s0}(V_{100})$ [μS cm^{-2}]	$\varepsilon[\lambda_\infty]$	$\varepsilon[N_p]$	$\varepsilon[\tau_s]$	$\frac{\delta[\lambda_\infty]}{\varepsilon[N_p]}$	$\frac{\delta[\tau_s]}{\varepsilon[\tau_s]}$
					$V_{200\,msec}$						
25 °C	1.0	6.53	21.6	2.63	33.4	8.0	4.0	3.2	0.9	2.9	2.9
	0.5	5.86	33.0	2.51	39.6	1.2					
					$V_{600\,msec}$						
18 °C	1.0	6.87	19.5	2.98	31.2	4.5	6.7	5.9	1.6	1.8	2.0
	0.5	6.29	37.4	2.87	40.8	0.50					
					$V_{1.8\,sec}$						
11 °C	1.0	7.08	22.5	3.80	31.8	3.4	7.9	7.1	1.8	1.6	2.1
	0.5	6.51	42.2	3.27	40.5	0.17					

[a] For definition of the $\alpha[i]$ and $\varepsilon[i]$ *see text*. V_{100} is the voltage at which $\lambda_\infty = 100$ μS cm^{-2}. The extrapolated initial conductance λ_{s0} is given at V_{100}. $\delta[\lambda_\infty]$ and $\delta[\tau_s]$ are taken from Table 2. Data are obtained under conditions of low λ_Φ-pretreatment.
Membrane solution: 1% di-(18:1)-lecithin in decane. Salt: KCl.
Antibiotic concentration: 5×10^{-7} g/ml alamethicin R_F30 in both compartments.

chose V_{100} to be this voltage. The mean values at a given temperature were taken for the calculation of $\alpha[\lambda_\infty]$.

The slow relaxation time τ_s has been evaluated correspondingly. The $\varepsilon[\tau_s]$ values defined in analogy to Eq. (7) were determined by comparing the voltages $V_{200\,msec}$ at 25 °C, $V_{600\,msec}$ at 18 °C and $V_{1.8\,sec}$ at 11 °C where τ_s amounts to 200 msec, 600 msec and 1.8 sec, respectively, at the different salt concentrations. For comparison of the magnitude of the weakly voltage-dependent conductance λ_{s0} values at V_{100} are also given in Table 3.

In view of the statement of Gordon and Haydon (1975) that the salt concentration dependence of alamethicin action is caused by an ionic strength effect on alamethicin adsorption to the membrane interface we studied the system at different salt concentrations but constant number of pores N_p. As will be outlined later and according to Eq. (9) the steady-state conductance λ_∞ is given by

$$\lambda_\infty = \left(\sum_v p_v \Lambda_v\right) \cdot N_p (t \to \infty).$$

Whereas the p_v distributions seem to be independent of salt concentration, $\Lambda_v \propto (C_{salt})^{0.73}$ was found by Eisenberg *et al.* (1973). This agrees with the

results of part II of this paper (Kolb & Boheim, 1977) that

$$\bar{A} = \sum_{\nu} p_{\nu} A_{\nu} \propto (C_{\text{salt}})^{0.8}.$$

Now, we introduce the exponential factor $\varepsilon[N_p]$ characterizing the salt concentration dependence of a characteristic voltage at constant N_p by $\varepsilon[N_p] = \varepsilon[\lambda_\infty] - \varepsilon[\bar{A}]$. The corresponding values with $\varepsilon[\bar{A}] = 0.8$ are listed in Table 3.

3. *Experiments under conditions of high λ_Φ-pretreatment (C) and temperature-pretreatment A.* In the second group of relaxation experiments the measurements were started after a steady state in the weakly voltage-dependent conductance was reached. This conductance value of about 100 μS cm^{-2} appeared to be nearly two orders of magnitude larger than the conductance observed after low λ_Φ-pretreatment for the given concentration of 2.5×10^{-7} g/ml alamethicin.

If the voltage is preset to 55 mV, where many pores are present in the system, an abrupt decrease of voltage by 10 mV reveals two current relaxations (Fig. 2*c*, *e*). The slow process (Fig. 2*c*) and the fast process (Fig. 2*e*) may be fitted by single exponential functions. For a check of the accuracy of the single exponential fitting, half-logarithmic plots of $(I - I_\infty)$ versus t have been drawn (Fig. 2*d*, *f*). Data of the fast relaxation process are obtained by a half-logarithmic plot of the difference between the experimental points and the extrapolated values of the slow process. Under the conditions given in Fig. 2*c* and *e* the two time constants differ by a factor of 240. The fast current-relaxation occurs in the ms-range which is of the same order of magnitude as the mean life-times τ_ν of single pore states. The same occurrence of two relaxation processes is observed after a 10-mV up-jump from 45 to 55 mV (Fig. 2*g*, *h*), because many pores are already existing at the initial voltage in this case, also. It is obvious that the slow relaxation (Fig. 2*g*) corresponds to the one observed in Fig. 2*a*.

Data given below have been obtained from 2 to 4 current-relaxation series at each temperature. In case of a single membrane which lasted longer than 20 hr the results obtained in the above-described sequence (temperature-pretreatment *A*) showed a reproducibility which was considerably better than the one observed for experiments under the conditions of low λ_Φ-pretreatment. The disadvantage of high λ_Φ-pretreatment is, however, that the membrane breaks in most cases during the preconditioning procedure.

The following quantities can be determined from the experimentally obtained relaxation curves (Fig. 2*c*, *e*, *g*, *h*): the fast relaxation time τ_f, the initial current $I(t=0)$ which is within experimental accuracy identical to the current I_{f0} obtained by extrapolation of the fast relaxation process to $t=0$, the slow relaxation time τ_s, the current I_{s0} obtained by extrapolation of the slow first-order relaxation process to $t=0$ and the final steady-state current I_∞. These values were obtained for a 10 mV down-jump and a 10 mV up-jump, whereby long-time pretreatment was done at the higher voltage.

Fig. 5*a* shows the conductance-voltage characteristic of an alamethicin-modified lipid membrane system under conditions of high λ_Φ-pretreatment at 11 °C. The appearance of a weakly voltage-dependent conductance of large magnitude can be seen. Furthermore, a saturation behavior occurs at conductances $\lambda_\infty > 3$–5 mS cm^{-2}. Whereas the weakly voltage-dependent conductance is nearly independent of temperature, the voltage-dependent conductance slightly decreases with increasing temperature and the saturation shifts to higher conductances.

The occurrence of first-order relaxation curves after the application of 10 mV pulses within the saturation range is still seen (Fig. 2*c*, *g*); at longer times an additional relaxation process of small amplitude is observed. On the other hand, if a voltage-jump from 20 to 55 mV under the conditions of Fig. 2*g* is applied, the current-relaxation curve shows a pronounced inactivation behavior, i.e. the current increases to a maximum value and then decreases with a time constant which is at least one order of magnitude larger than the relaxation time τ_s. This is different in the suzukacillin system where activating and inactivating processes are of the same time scale (Boheim *et al.*, 1976). The appearance of such an inactivation at large alamethicin-induced conductances had already been observed by Mueller (1976*b*). The conductance value at the maximum in the current-relaxation curve after a voltage-jump from 20 to 55 mV approaches the value given by straight line extrapolation in Fig. 5*a*.

Values of the $\alpha[\lambda_\infty - \lambda_\Phi]$-parameter are given in Table 4. For its determination the final conductances $\lambda_\infty(V)$ had to be corrected for the weakly voltage-dependent conductance $\lambda_\Phi(V)$. λ_Φ is defined by the conductance of 'voltage-independent' pores which exist at zero voltage and are not closed by the application of negative voltages. After the application of positive voltages within the strongly voltage-dependent range of λ_∞ their conductance increases slightly with voltage as has been demonstrated in the previous section. If 'voltage-dependent' pores exist at zero voltage, they are closed by the application of negative voltages. Because

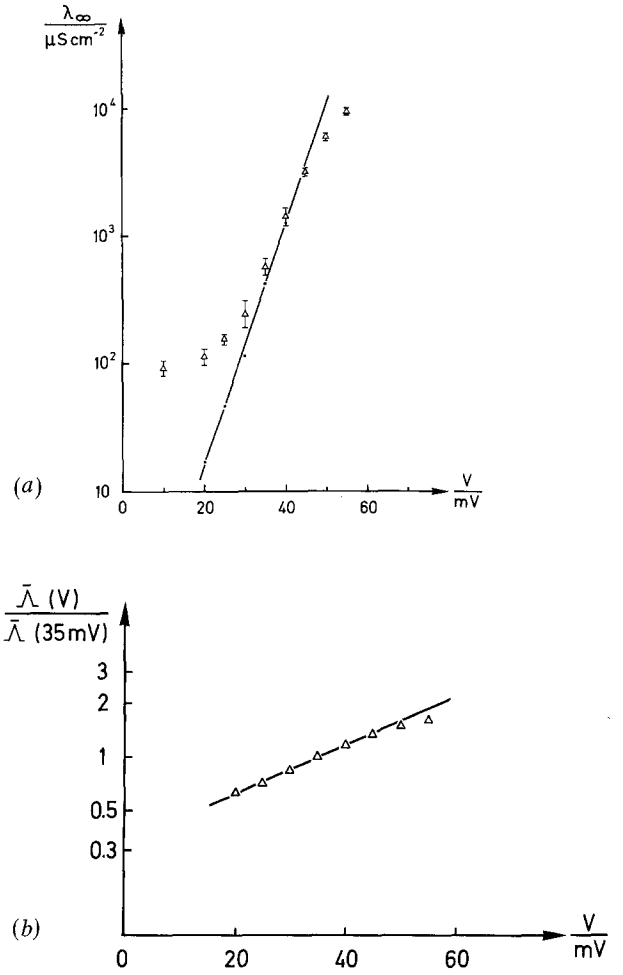

Fig. 5. (*a*) Half-logarithmic plot of the final conductance $\lambda_\infty(\triangle)$ versus voltage V of an alamethicin-modified lipid membrane under conditions of high λ_Φ-pretreatment. The full points (●) through which the straight line is drawn are obtained by subtracting the weakly voltage-dependent conductance λ_Φ from λ_∞. (*b*) Relative change of the mean pore conductance $\bar{\Lambda}$ with voltage V in a half-logarithmic plot. (For details *see text.*) Membrane solution: 1% di-(18:1)-lecithin in decane. Salt solution: 1 M KCl, unbuffered. Antibiotic concentration: 2.5×10^{-7} g/ml alamethicin R_F30 in both compartments. Temperature: 11 °C

their conductance is still an exponential function of voltage, a negative differential part in the current-voltage characteristic occurs which was demonstrated by Eisenberg *et al.* (1973).

The values of λ_Φ at 10 mV are given in Table 4. Their voltage-dependence is assumed to be approximated by the voltage-dependence of the mean pore conductance $\bar{\Lambda}(V)$ *(see below).* If the initial voltage before a

Table 4. Values of the final conductance λ_∞, the slow relaxation time τ_s, the fast relaxation time τ_f, the ratio λ_∞/τ_s and of the voltage dependence characterizing parameters $\alpha[\lambda_\infty - \lambda_\Phi]$, $\alpha[\tau_s]$, $\alpha[\tau_f]$, $\alpha[\lambda_\infty/\tau_s]$ at different temperatures[a]

		32 °C	25 °C	18 °C	11 °C
λ_∞	V_{1000}/ [mV]	41.6	40.0	37.8	38.0
	$\alpha[\lambda_\infty - \lambda_\Phi]$	5.35	5.28	5.12	5.31
	λ_Φ(10 mV)/ [μS cm^{-2}]	101	109	90	92
$\bar{\Lambda}$	$\alpha[\bar{\Lambda}]$	0.93	0.95	0.89	0.79
τ_s	τ_s(40 mV)/ [sec]	0.021	0.075	0.33	1.5
	$\alpha[\tau_s]_{dp}$	2.27	2.44	2.51	2.73
	$\alpha[\tau_s]_{ip}$		3.11	3.22	3.70
τ_f	τ_f(40 mV)/ [msec]	1.8	3.1	4.6	8.4
	$\alpha[\tau_f]$		0.18	0.18	0.18
$\frac{\lambda_\infty}{\tau_s}$	$\frac{\lambda_\infty}{\tau_s}$ (40 mV)/ [mS cm^{-2} sec^{-1}]	31	15	4.5	0.96
	$\alpha\left[\frac{\lambda_\infty}{\tau_s}\right]_{dp}$	3.45	2.66	2.21	1.96
	$\alpha\left[\frac{\lambda_\infty}{\tau_s}\right]_{ip}$		2.5	1.83	1.01
μ	$\alpha[\mu]_{dp}$	2.5	1.7	1.3	1.2
	$\alpha[\mu]_{ip}$	2.5	1.5	0.9	0.2

[a] $\alpha[\bar{\Lambda}]$, $\alpha[\mu]$ are the corresponding parameters of the mean pore conductance $\bar{\Lambda}$ and of the pore formation rate μ. In addition, the values of the weakly voltage-dependent conductance λ_Φ at 10 mV are listed. For details *see text*. Data are obtained under conditions of high λ_Φ-pretreatment.
Membrane solution: 1% di-(18:1)-lecithin in decane.
Salt solution: 1 M KCl, unbuffered.
Antibiotic concentration: 2.5×10^{-7} g/ml alamethicin R_F30 on both sides.

voltage-jump lies within the voltage-dependent conductance range, the extrapolated conductance λ_{s0} after the voltage-jump is not identical with λ_Φ. In this case additional "voltage-dependent" pores were present at the initial voltage. The $\lambda_\infty - \lambda_\Phi$ values have been indicated by full points in Fig. 5*a*. From the slope of the straight line drawn through these points the $\alpha[\lambda_\infty - \lambda_\Phi]$-parameter is obtained. Table 4 shows that $\alpha[\lambda_\infty - \lambda_\Phi]$ seems not to be temperature-dependent.

We want to characterize the conductance-voltage curve by a voltage within the exponential range of voltage-dependence. In Table 4 the

voltage V_{1000} has been introduced at which $\lambda_\infty = 1\ \mathrm{mS\ cm^{-2}}$, since the earlier defined V_{100} can only be obtained by extrapolation in this case.

The relative change of the mean pore conductance $\bar{\Lambda}$ with voltage is demonstrated in Fig. 5*b*. Following the theoretical description of Boheim *et al.* (1976) the multi-pore conductance per unit area λ is described by

$$\lambda = \frac{I}{V} = \bar{\Lambda} \cdot N_p \tag{9}$$

with

$$\bar{\Lambda} = \sum_v p_v \Lambda_v$$

where N_p is the total concentration of pores per unit area, $\bar{\Lambda}$ the mean pore conductance, Λ_v the conductance of the pore in state v and p_v the probability to find a pore in state v. The slow relaxation process is attributed to the variation of N_p with time. Therefore the extrapolated conductance $\lambda_{s0}(V_f)$ should result from the same number of pores as $\lambda_\infty(V_i)$, where V_i and V_f are the initial voltage before and the final voltage after the voltage-jump, respectively. With $N_p = \text{const.}$ the relation

$$\frac{\lambda_\infty(V)}{\lambda_{s0}(V \pm 10\ \mathrm{mV})} = \frac{\bar{\Lambda}(V)}{\bar{\Lambda}(V \pm 10\ \mathrm{mV})} \tag{10}$$

holds for a voltage-jump from V to $V \pm 10$ mV. Absolute $\bar{\Lambda}$ values cannot be determined by these relaxation measurements (in contrast to the autocorrelation analysis, *see* part II). Thus, changes relative to $\bar{\Lambda}$ (35 mV) have been considered in Fig. 5*b*. There the mean value of $\bar{\Lambda}$ for jumps from V to $V+10$ mV and from $V+10$ mV to V has been taken as $\bar{\Lambda}(V+5\ \mathrm{mV})$. According to Table 4, values of the parameter $\alpha[\bar{\Lambda}]$ between 0.79 and 0.95 are found. $\alpha[\bar{\Lambda}]$ was obtained from the slope of straight lines (as in Fig. 5*b*), whereby the deviation at higher voltages was not taken into account.

The τ_s values have been plotted in Fig. 6*a* as a function of the final voltage. The plot shows that a down-jump from $V+10$ mV to V (prolonged-time preconditioning at $V+10$ mV; $\times$ 25 °C, $\triangle$ 11 °C) yields a faster relaxation time than an up-jump from $V-10$ mV to V (prolonged-time preconditioning at V, short-time preconditioning at $V-10$ mV; $\otimes$ 25 °C, $\blacktriangle$ 11 °C). The time constants differ by a factor of about 1.5. If in both cases the prolonged-time preconditioning was done at *the same voltage* V, these τ_s values approximately became identical. This observation had also been reported by Roy (1975) for membranes made from egg lecithin. Thus the differences in the τ_s values which were measured

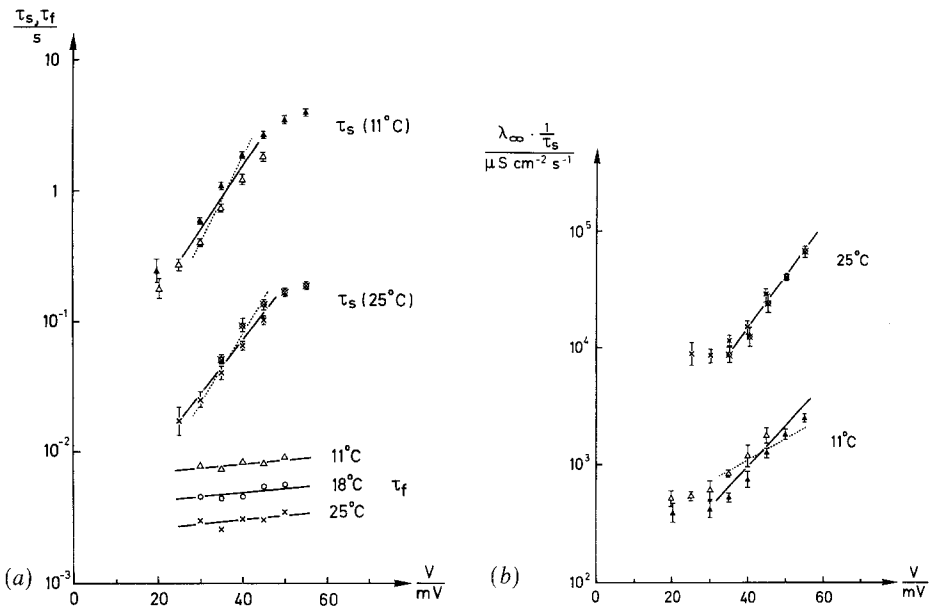

Fig. 6. (*a*) Half-logarithmic plot of the slow relaxation time τ_s and the fast relaxation time τ_f versus voltage V of an alamethicin-modified lipid membrane under conditions of high λ_Φ-pretreatment. The data have been plotted as a function of the final voltage *V*. The upper points of τ_s (▲, 11 °C; ⊗, 25 °C) represent voltage-jumps from $V - 10$ mV to *V*, whereby prolonged-time preconditioning occurred at *V*. The lower points of τ_s (△, 11 °C; ×, 25 °C) represent voltage-jumps from $V + 10$ mV to *V*, whereby prolonged-time preconditioning was done at $V + 10$ mV. The τ_f values (△, 11 °C; ○, 18 °C; ×, 25 °C) turned out to be independent of voltage-preconditioning. (For details and explanation of the drawn straight lines *see text.*) (*b*) Ratio λ_∞/τ_s of the final conductance λ_∞ and the slow relaxation time τ_s which is related to the pore formation rate μ by $\lambda_\infty/\tau_s = \mu \cdot \bar{A}$ in a half-logarithmic plot versus voltage *V*. The data are plotted in the same manner as outlined under (*a*). Experimental conditions of this Figure are the same as for Fig. 5

at the same final voltage seem primarily to result from the different voltages of prolonged-time preconditioning.

τ_s (Fig. 6*a*) shows a saturation behavior comparable to that of λ_∞ (Fig. 5*a*). Within the approximately linear region between 30 and 45 mV for up-jumps and down-jumps, two straight lines have been drawn. The solid line is the mean line between the straight connections of the upper experimental points (up-jumps) and of the lower points (down-jumps), respectively. Its slope $\alpha[\tau_s]_{dp}$ describes the voltage dependence of τ_s, if the voltage of prolonged-time preconditioning is changed with the measuring voltages (*dp* means different preconditioning). $\alpha[\tau_s]_{dp}$ is listed in Table 4 and decreases slightly with increasing temperature. A second, broken line is introduced in Fig. 6*a* which would yield the voltage depen-

dence of τ_s for identical prolonged-time preconditioning at a given voltage. The corresponding $\alpha[\tau_s]_{ip}$ are also listed in Table 4 (*ip* means identical preconditioning). The mean difference between the τ_s values at 40 mV for a temperature difference of 7 °C is about a factor of 4. This corresponds to an activation energy of 34 kcal/mol. In the case of different voltages of prolonged-time preconditioning this value is nearly independent of voltage.

The fast relaxation time τ_f is found in the 1 to 10 ms-range (Fig. 6*a*); it shows only a weak voltage dependence (Table 4). Deviations from the given mean τ_f of $\pm 20\%$ were observed. Within these limits of reproducibility the τ_f values did not show a dependence on the voltage of prolonged-time preconditioning. The difference between the τ_f values is about a factor of 1.7 for a temperature change of 7 °C. This corresponds to an activation energy of about 12 kcal/mol, which is nearly voltage independent.

The initial conductance $\lambda(t=0)$ showed ohmic behavior for 10 mV jumps up to 45 mV. A weak superlinear increase of 2.5% was observed for voltage-jumps from 40 to 50 mV and of 6% for up-jumps from 45 to 55 mV. The same effect occurred at the corresponding down-jumps.

As will be shown in a later section [Eq. (21)] the relation

$$\frac{\lambda_\infty}{\tau_s} = \mu \cdot \bar{A} \tag{11}$$

holds, whereby μ is the pore formation rate. In order to investigate the properties of μ we consider the ratio λ_∞/τ_s. Fig. 6*b* shows that λ_∞/τ_s exhibits a weak voltage dependence at low voltages and an exponential dependence in the 40 to 55 mV-range in contrast to τ_s. The ratio of two λ_∞/τ_s values taken at temperatures which differ by 7 °C varies with the mean temperatures. This indicates that the activation energy depends on temperature. Between 18 and 25 °C a mean activation energy of 29 kcal/mol is found. The extent of the weak voltage dependence at low voltages is comparable to that of $\bar{A}$.

Between 40 and 55 mV two straight lines have been drawn in Fig. 6*b* again according to the different procedures of prolonged-time preconditioning. The solid line is the mean line between the straight connections of the upper experimental points (down-jumps) and of the lower points (up-jumps), respectively. The $\alpha[\lambda_\infty/\tau_s]_{dp}$ values obtained from that slope under the conditions of prolonged pretreatment at different voltages are listed in Table 4. They increase with increasing temperature. The broken line would represent the voltage dependence of λ_∞/τ_s at a given voltage and identical prolonged-time preconditioning. The $\alpha[\lambda_\infty/\tau_s]_{ip}$ values are

also given in Table 4. They are smaller than the $\alpha[\lambda_\infty/\tau_s]_{dp}$ values in contrast to the situation with τ_s and approach those at higher temperatures. The $\alpha[\mu]$-parameter is given by [compare Eq. (5)]

$$\alpha[\mu]=\alpha[\mu\bar{\Lambda}]-\alpha[\bar{\Lambda}]=\alpha[\lambda_\infty/\tau_s]-\alpha[\bar{\Lambda}]. \tag{12}$$

Estimates of its values are added in Table 4.

Discussion

Basic Model Considerations

It has been stated that alamethicin pore fluctuations appear in bursts (Gordon & Haydon, 1972, 1975; Eisenberg *et al.*, 1973). Within one burst usually 5 to 6 conductance levels appear which are adopted in a consecutive order. This peculiar sequence is visible because the conductance levels are nonintegral multiples of each other. This has been interpreted in such a way that each burst represents an active alamethicin aggregate (pore) which can adopt several conductance states.

Fig. 1*b* shows that the mean life-times τ_ν of the three most probable pore states are nearly identical. In addition it has been demonstrated for di-(22:1)-lecithin membranes (Boheim, 1974) and also for di-(18:1)-lecithin membranes (Boheim, *unpublished results*) that the mean life-time $\tau_{\nu'}$ of the most probable pore state ν' is nearly voltage-independent although ν' changes with voltage. According to Eq. (14) in Boheim (1974) $\tau_{\nu'}$ is determined by

$$\tau_{\nu'}=\frac{1}{k^*_{\nu',\nu'+1}+k_{\nu',\nu'-1}}. \tag{13}$$

A comparison between the fast relaxation time τ_f observed in multipore experiments and the mean life-time $\tau_{\nu'}$ obtained from single-pore experiments shows that there is strong evidence for the identity of the underlying molecular process in both cases: (1) Only a weak voltage dependence is found for τ_f in contrast to the relative strong voltage dependence of τ_s. (2) An activation energy of about 12 kcal/mol is calculated for both time constants. This agrees with the results of Gordon and Haydon (1976) which give an activation energy of about 10 kcal/mol for the rate constants of pore state transitions.[2] (3). Both time constants are

2 The activation energy in the paper of Gordon and Haydon (1976) has been erroneously given to be 1.2 kcal/mol (D. A. Haydon, *personal communication*).

of the same order of magnitude in time. The difference by a factor of 4 in the absolute values of $\tau_{v'}$ (100 mV) and τ_f (40 mV) at 11 °C cannot be attributed to the different voltages. As discussed in more detail later, the difference may be caused by different properties of the membrane lipids in the single-pore or multi-pore case according to different preconditioning procedures.

In view of the good approximation of the ensemble of pore state relaxations by a single time constant, it seems possible to replace Eq. (A.1) by a two-state equation. Under single-pore conditions about 70% of its life-time the pore adopts one of the two most probable pore states v', $v'+1$ in lecithin membranes for a voltage interval of about 20 mV (Boheim, 1974). We therefore introduce a mean pore state $\bar{v}$ (Boheim, 1974) defined by

$$\bar{v} = \sum_v p_v \cdot v \tag{14}$$

and assume that $\bar{v} - \frac{1}{2} = j$ and $\bar{v} + \frac{1}{2} = j+1$ are the two most probable pore states under multi-pore conditions. If $N_p = \sum_v N_v$ is the total concentration of pores (mol cm^{-2}) we introduce the approximation $N_p = N_j + N_{j+1}$. Thus we obtain instead of Eq. (A.1)

$$\frac{dN_j}{dt} = k_{j+1,j} \cdot N_p - (k^*_{j,j+1} + k_{j+1,j}) \cdot N_j. \tag{15}$$

Under the assumption of $N_p = \text{const.}$ the time constant τ_f of this first-order equation is given by

$$\tau_f = \frac{1}{k^*_{j,j+1} + k_{j+1,j}} \tag{16}$$

which is similar to Eq. (13).

We identify the slow relaxation time τ_s observed in multi-pore experiments with the mean life-time of a fluctuating burst for the following reasons. (1) The bursts usually show a mean life-time which is at least one or two orders of magnitude larger than the mean life-time of the pore states. The same is observed with τ_s and τ_f. (2) The results of the autocorrelation analysis (Kolb & Boheim, 1977) show that the absolute value and voltage dependence of the single-unit conductance obtained from the correlation amplitude of the slow process are comparable with the corresponding quantities of the mean pore conductance $\bar{A}$ as expected from single-pore analysis.

Table 1 indicates that τ_s appreciably depends on voltage and alamethicin concentration. Eq. (A.6) describes the fundamental relation between single-pore and multi-pore phenomena. Therefore, we consider the voltage and alamethicin concentration dependence of τ_s with respect to that of p_0. The result is given in the Appendix. On the basis of functional analytical arguments a factor ρ has been defined by [*see* Eq. (A.17)]

$$\rho = \frac{\alpha}{x} = \frac{\alpha[\tau_s]}{\delta[\tau_s]}. \tag{17}$$

x is the number of molecules which are involved in a pore state transition. α is the fraction of the equivalent of one positive elementary charge transferred across the membrane during a transition to the next higher pore state.

As shown in Table 2 the presented multi-pore experiments yield values of $0.91 \leqq \rho \leqq 0.98$ between 4 and 25 °C. From the statistical analysis of a single fluctuating pore at different voltages $\alpha = 0.93$ was obtained (Boheim, 1974). According to Eq. (17) this estimates x to be of the order of 1. Consequently, Γ [Eqs. (A.7), (A.15)] is a linear function of N_s and the subunit which is uptaken or released from the pore during each pore state transition seems to be a single alamethicin molecule.

We wish to emphasize that we obtained this result without assumption of a peculiar molecular pore model. It follows immediately from the phenomenological behavior of the alamethicin single-pore and multi-pore systems as a consequence of the fact that the mean life-time of a fluctuating pore is determined by the dissociation rate of its lowest state *and* by the probability of the pore to adopt that state. This result puts strong restrictions on each molecular model for the alamethicin pore formation mechanism.

A second fact which is obtained from Eqs. (A.6), (A.12) and (A.13) concerns an estimate of the mean pore state $\bar{\nu}$. The value $\nu = \nu'$ (ν' is an integral number) of the largest term of the sum in Eq. (A.9) is characterized by $K^*_{\nu'} \gtrsim 1$, whereby ν' is situated near $a[1/p_0]$. If we assume that the p_ν distributions are symmetric, as found by Boheim (1974) and Gordon and Haydon (1976), then in the particular case of $K^*_{\nu'} = 1$ which, according to Eq. (A.7), corresponds to $p_{\nu'} = p_{\nu'-1}$ the mean pore state $\bar{\nu}$ would be given by $\bar{\nu} = \nu' - \frac{1}{2}$. $\bar{\nu}$, which is defined by Eq. (14), usually is not an integral number. Consequently, an estimate of $\bar{\nu}$ may be obtained from the voltage and alamethicin concentration (or Γ) dependence, respectively, of τ_s (and $1/p_0$) by

$$\bar{\nu} \approx a - \tfrac{1}{2}. \tag{18}$$

This relation is confirmed for the case of single-pore statistics (Boheim, 1974). We obtain from Fig. 8*b* $\delta[1/p_0]=a[1/p_0]=3.3$ and thus $\bar{\nu}\approx 2.8$ at $V=60$ mV. The data of the statistical analysis (Boheim, 1974) yield $\bar{\nu}\approx 3.0$ at 63 mV.

The notion that the slow relaxation time τ_s should be approximately equal to the mean life-time τ_b of the pore [Eq. (A.6)] can be checked, in principle, by a direct determination of τ_b from single-pore statistics. Such a comparison has not yet been carried out under the conditions of our experiments. Gordon and Haydon (1976) have measured τ_b at different voltages for monooleate-cholesterol-hexadecane membranes. The results of their experiments yield a value of $\alpha[\tau_b]\approx 0.4$–$0.5$ [estimated from Fig. 3 of Gordon and Haydon (1976)], which is much smaller than our value of $\alpha[\tau_s]$. The discrepancy between $\alpha[\tau_b]$ and $\alpha[\tau_s]$ probably arises from the fact that Gordon and Haydon analyzed the pore statistics at an approximately constant pore formation rate μ, i.e., at a nearly constant frequency of appearance of bursts. For this purpose they had to compensate an increase of voltage by a corresponding decrease of alamethicin concentration. In view of the voltage-alamethicin concentration equivalence for τ_s [Eq. (A.13)] their finding of a voltage independence of τ_b has to be expected. Under the same experimental conditions Gordon and Haydon (1975, 1976) recorded the probability distribution of the pore states p_ν. Within experimental accuracy they found p_ν to be nearly voltage-independent, which probably also results from a mutual compensation of the effects of changes in voltage and alamethicin concentration. Their finding that neither τ_b nor p_0 is voltage-dependent under their experimental conditions is consistent with the equation $\tau_b=1/p_0 k_{0m}$ which corresponds to Eq. (A.6), because k_{0m}, too, is likely to be voltage-independent (*see* Appendix).

The same arguments are applicable to the experimental data of Eisenberg *et al.* (1973) and Hall (1975), who did not find a voltage dependence of the probabilities p_ν but a voltage dependence of $\alpha[\tau_s]\approx 2.7$ for the (slow) relaxation time τ_s of a multi-pore system.

Boheim (1974) carried out a statistical analysis of single pores which did not stop fluctuating while the applied voltage was changed. An equivalent method was used by Mueller (1976*a*) and Gordon and Haydon (1976) who applied a voltage-jump to a single fluctuating pore and compared the p_ν values at the two different voltages. Whereas Mueller (1976*a*) reported a virtually immediate (within 10 msec) shift in p_ν after voltage-jumps of 40–70 mV amplitude, Gordon and Haydon (1976) observed no change with voltage-jumps of 10 mV which is probably within the limits of sensitivity of the experiments.

A second difference in the experimental conditions of the single-pore measurements of Gordon and Haydon (1975, 1976), Eisenberg *et al.* (1973) and Hall (1975) on one side and Boheim (1974) on the other results from the different temperatures. Whereas Boheim (1974) measured at low temperature (3–11 °C), the other experiments were carried out at room temperature (20–23 °C). The data presented in this paper show that long-lasting pores which may change their state about 10^3–10^4 times before closing have a much higher probability to appear at lower temperatures. Fig. 6*a* indicates that the ratio τ_s/τ_f which is a measure of the mean number of events during one burst changes from about 25 at 25 °C to 200 at 11 °C at 40 mV, i.e., one order of magnitude as a consequence of the different activation energies of τ_s and τ_f. In view of this finding one has to conclude that the analyses of Eisenberg *et al.* (1973), Hall (1975) and Gordon and Haydon (1975, 1976) were done at a large number of relatively short-living pores.

The arguments presented above seem to indicate that the correct value of α is close to 0.9. What could be the nature of this voltage dependence for the activation of a single alamethicin molecule? Eisenberg (1972) as well as Mauro, Nanavati and Heyer (1972) have given experimental evidence that the alamethicin pore exhibits an intrinsic asymmetry. Pores were formed with a slow relaxation time after application of a large positive voltage in a system which was symmetrical with respect to all components. An abrupt jump to a negative voltage of same size led to an immediate closing of the existing pores and to the formation of new pores again with a slow relaxation time. Subsequent switching to the initial voltage showed the same effect. This behavior is different from the nearly quadratic voltage dependence of gramicidin A pore formation (Bamberg & Benz, 1976). The voltage effects on alamethicin could be caused by various effects, for instance by the transport of weakly bound cations across the membrane or the orientation of permanent dipoles from the interface into the membrane. A further possibility would be a voltage-triggered conformational change from a structure corresponding to a more hydrophilic environment to a structure peculiar to the conducting channel. At the moment no detailed explanation of the voltage dependence of alamethicin action can be given. Therefore we only introduce an equivalent charge of about 0.9 elementary charges which should account for the voltage dependence if it is assumed that this charge is transferred across the membrane during a pore state transition to the next higher state. The finding of $x \approx 1$ indicates that one equivalent charge is associated with one alamethicin molecule.

Gordon and Haydon (1972, 1975) proposed that the alamethicin pore consists of a bunch of parallel channels. Their considerations are consistent with the notion that only one alamethicin molecule is responsible for a pore-state transition. However, in view of the recently proposed linear structure of alamethicin (Jung *et al.*, 1975; Martin & Williams, 1975, 1976), where a part of the molecule adopts α-helical structure, a channel created by only one molecule appears to be quite unlikely. Hall (1975), on the other hand, proposed the model of a two-dimensional micelle which once pushed into the membrane might fluctuate between various conformations. In this model the transitions to more or less open configurations are caused by interactions between several molecules within the micelle. However, the experimental results presented in this paper indicate that each pore-state is in a distribution equilibrium with the interfacial alamethicin monomer concentration and that a steady uptake and release of monomers ($x \approx 1$) from the fluctuating pore occurs. Therefore, all molecular models which assume an active pore aggregate of fixed size, where the different conductance steps result from conformational changes of this aggregate (Hall, 1975; Gordon & Haydon, 1976; S.I. Chan, *personal communication*), and which imply $x=0$ are inconsistent with the experimental observations that τ_s strongly depends on the alamethicin concentration and that a strict voltage-alamethicin concentration relation exists.

Initial Conductances of the Fast and of the Slow Relaxation Process

The relation between the current I and the pore concentration N_p is given by Eq. (9):

$$I = \bar{\Lambda} \cdot N_p \cdot V = \sum_{\nu=0}^{\infty} (p_\nu \Lambda_\nu) \cdot N_p \cdot V.$$

This equation immediately shows the separation into the term of fast relaxation (p_ν) and the term of slow relaxation (N_p).

The initial conductance λ_{f0} after a voltage-jump from V_1 to V_2 is given by

$$\frac{I(t=0)}{V_2} = \lambda_{f0}(V_2) = \sum_{\nu=0}^{\infty} (p_\nu(V_1) \cdot \Lambda_\nu(V_2)) \cdot N_p(V_1). \tag{19}$$

λ_{f0} is ohmic up to 45 mV under the experimental conditions of Figs. 5 and 6 and shows a slight nonlinear increase beyond this voltage. According to Eq. (19) this should result from a voltage-dependence of Λ_ν, which

indeed had been observed in single-pore measurements (Haydon *et al.*, 1972; Eisenberg *et al.*, 1973; Boheim, 1974).

From current-relaxation measurements we cannot determine the absolute value of $\bar{\Lambda}$. Therefore only its voltage dependence in terms of the parameter $\alpha[\bar{\Lambda}]$ is considered. From single-pore fluctuation analysis of alamethicin in di-(22:1)-lecithin membranes (Boheim, 1974) $0.3 \leqq \alpha[\bar{\Lambda}] \leqq 0.5$ is obtained, $\alpha[\bar{\Lambda}]$ becoming smaller at higher mean pore states. If at the voltage V_1 a few pores are already existing in the system, the conductance λ_{s0} obtained by extrapolation of the slow relaxation process after a voltage-jump to V_2 is given by

$$\frac{I_s(t=0)}{V_2} = \lambda_{s0}(V_2) = \bar{\Lambda}(V_2) \cdot N_p(V_1). \tag{20}$$

With

$$\lambda_\infty(V_1) = \bar{\Lambda}(V_1) \cdot N_p(V_1)$$

and $V_1 = V$ and $V_2 = V \pm 10$ mV, Eq. (10) is obtained.

In the first series of relaxation experiments positive voltage-jumps from $V_1 = 0$ mV to $V_2 > 0$ were recorded, whereas in the second series positive and negative jumps between different voltages $V_1 > 0$ and $V_2 > 0$ have been carried out. In both cases mean values of $\alpha[\bar{\Lambda}]$ between 0.75 ± 0.1 and 0.95 ± 0.1 are obtained, whereby $\alpha[\bar{\Lambda}]$ seems to be larger at higher temperatures. Similar values have been observed with suzukacillin (Boheim *et al.*, 1976). These $\alpha[\bar{\Lambda}]$ values are by about a factor of 2 larger than the values obtained from single-pore experiments. The deviation is equivalent to a 20% shift in the experimental λ_{s0} values. On the other hand, $\alpha[\bar{\Lambda}]$ values determined by autocorrelation analysis of a multi-pore system match the single-pore values quite well (Kolb & Boheim, 1977).

The larger $\alpha[\bar{\Lambda}]$ values obtained by voltage-jump experiments may be caused by deviations from the presented simple reaction scheme assumed here. With single-pore experiments the number of short-lived pores is larger than expected from an exponential distribution of life-times (Gordon & Haydon, 1975). In terms of Eq. (9) this effect is equivalent to an overshoot in the p_v distributions immediately after the voltage-jump, which would be an explanation for the larger $\alpha[\bar{\Lambda}]$ values.

Steady-State Conductance and Slow Relaxation Time

The steady-state conductance λ_∞ is obtained from Eqs. (9), (A.5) and (A.6):

$$\frac{I(t \to \infty)}{V} = \lambda_\infty = \bar{\Lambda} \cdot N_p(t \to \infty) = \bar{\Lambda} \cdot \mu \cdot \tau_s,$$

where

$$N_p(t\to\infty)=\mu\cdot\tau_s \tag{21}$$

is the steady-state solution ($dN_p/dt=0$) of Eq. (A.5). The voltage and alamethicin concentration dependence of λ_∞ and τ_s is summarized in Tables 1, 2 and 4. $\alpha[\lambda_\infty]$ and $\alpha[\tau_s]$ are independent of alamethicin concentration but depend on temperature and membrane pretreatment. A comparison of the $\alpha[\tau_s]$ values of Table 1 with $\alpha[\tau_s]_{dp}$ and $\alpha[\tau_s]_{ip}$ of Table 4 indicates that experiments under conditions of low λ_Φ- and high λ_Φ-pretreatment seem to give similar results if in the latter case long-time preconditioning has been applied at identical voltages. The $\alpha[\tau_s]_{dp}$ values might be influenced by the different preconditioning voltages. The state of the alamethicin multi-pore system after application of a voltage-jump is different if the system is considered at shorter times (after 1–10 sec) or at longer times (>1 min).

In order to discuss the dependence of the properties of an alamethicin-modified lipid membrane system on alamethicin concentration and temperature we have to consider critically the procedure of data coordination. We characterized the voltage-dependent part of the current-voltage characteristic at a given alamethicin concentration, salt concentration and temperature by the characteristic voltages V_{100} and V_{1000} at which $\lambda_\infty=100\ \mu S\ cm^{-2}$ and $\lambda_\infty=1\ mS\ cm^{-2}$, respectively, and by the parameter $\alpha[\lambda_\infty]$ obtained from its slope. A shift of the current-voltage curve with changing alamethicin concentration or temperature then was evaluated in terms of V_{100} (V_{1000}) and $\alpha[\lambda_\infty]$. This procedure is equivalent to that of observing changes in λ_∞ at constant voltage V. It seems to be appropriate to compare systems under conditions of identical pore concentrations N_p and identical pore state distributions p_ν. Then according to Eq. (9) the analysis on the basis of identical λ_∞ values, as carried out in this paper, is only possible if $\bar{\Lambda}=\sum_\nu p_\nu\Lambda_\nu$ is independent of alamethicin concentration and temperature at the given λ_∞ value. Within the experimentally considered range $\alpha[\lambda_\infty]$ and $\alpha[\tau_s]$ turned out to be virtually independent of alamethicin concentration. This indicates, according to Eqs. (A.16) and (18) that the same applies to the mean pore state $\bar{\nu}$. Moreover, the absolute value of τ_s remains approximately unchanged with varying alamethicin concentration at constant λ_∞ (*see also* Kolb & Boheim, 1977) which implies $p_0\approx$ const. according to Eq. (A.6). Both arguments lead to the conclusion that at a given salt concentration the p_ν distributions and thus $\bar{\Lambda}$ are virtually independent of alamethicin concentration at $\lambda_\infty=$ const. The exact evaluation shows, however, that the system

is not unique at a constant λ_∞ but varying alamethicin concentration and voltage with respect to p_v and N_p as a consequence of the different ratios $\alpha[\lambda_\infty]/\delta[\lambda_\infty]$ and $\alpha[\tau_s]/\delta[\tau_s]$. Fig. 1*a* shows that the single-pore state conductances Λ_v decrease with decreasing temperature. On the other hand, it can be seen from Table 1 that $\alpha[\tau_s]$ and, according to Eqs. (A.16) and (18), the mean pore state $\bar{v}$ increases with decreasing temperature. Consequently also the p_v distributions change. Additional information is given by the autocorrelation analysis (Kolb & Boheim, 1977), where absolute $\bar{\Lambda}$ values have been determined. There it is shown that Λ_v and p_v change in such a way that $\bar{\Lambda}$ remains virtually independent of temperature. In view of the above given arguments it seems reasonable to chose as reference values $\lambda_\infty = 100\,\mu\text{S cm}^{-2}$ (low λ_Φ-pretreatment) or $\lambda_\infty = 1\,\text{mS cm}^{-2}$ (high λ_Φ-pretreatment), respectively.

Both the $\alpha[\lambda_\infty]$ and $\delta[\lambda_\infty]$ values decrease with increasing temperature. The results of Mueller and Rudin (1968) of $\alpha[\lambda_\infty] = 5.5$ and $\delta[\lambda_\infty] = 6$ at 35 °C, therefore, are consistent with our data obtained under conditions of low λ_Φ-pretreatment. $\alpha[\tau_s]$ and $\delta[\tau_s]$ show equivalent changes with temperature, leaving the ratio $\rho = \alpha[\tau_s]/\delta[\tau_s]$ [Eq. (17)] nearly temperature-independent. As a consequence of the dependence of τ_s on the p_v distributions the calculated activation energy $E_A[\tau_s]$ is not that of an elementary process in contrast to the situation with τ_f (taking into account our simplifying assumptions) and τ_v, respectively. $E_A[\tau_s]$ depends on the voltage and the pretreatment of the membrane. In case of low λ_Φ-pretreatment and 2.5×10^{-7} g/ml alamethicin $E_A[\tau_s] \approx 26$ kcal/mol at 50 mV and $E_A[\tau_s] \approx 34$ kcal/mol at 60 mV is obtained (Fig. 3*c*). In case of high λ_Φ-pretreatment at the same alamethicin concentration $E_A[\tau_s] \approx 34$ kcal/mol results, nearly independent of membrane voltage (Fig. 6*a*). A rough estimate of the pore nucleus decay rate k_{0m} at 11 °C is possible by setting $\bar{v} \approx 3.4$ for the mean pore state [obtained from $\delta[\tau_s]$ of Table 2 and Eq. (18)] and thus $p_0 \approx 2 \times 10^{-4}$ according to the p_v distribution set from Boheim (1974). With Eq. (A.6) and $\tau_s = 1.8$ sec a value of $k_{0m} \approx 3 \times 10^3\,\text{sec}^{-1}$ is obtained in case of the multi-pore system.

Pore Formation Rate

The properties of the pore formation rate μ are determined according to Eq. (11) by λ_∞, τ_s and $\bar{\Lambda}$. The validity of Eqs. (5) and (12) are evident taking into consideration the definition of the $\alpha[i]$-parameters. To account for the alamethicin concentration dependence of $\bar{\Lambda}$ at constant

voltage a value of $\delta[\bar{A}] \approx 1$ was introduced in Eq. (6). Since the voltage-alamethicin concentration relation [Eq. (17)] should be valid in this case, too, $\delta[\bar{A}] \approx 1$ follows from the experimental ρ- and $\alpha[\bar{A}]$-data.

An alternative method to determine the pore formation rate μ is to count directly the number of bursts per cm^2 per second under single-pore conditions in the absence of overlapping occurrences. A rough estimate using the data from Fig. 9 of Gordon and Haydon (1975) yields $\alpha[\mu] \approx 3$ at 20 °C which may be compared with the data of Table 1 obtained under conditions of low λ_Φ-pretreatment. Both methods give comparable results.

A second important result, besides the ρ relation [Eq. (17)] of τ_s which implies that the equivalence of about one positive elementary charge is associated with one alamethicin molecule, is obtained from the voltage and alamethicin concentration dependence of the pore formation rate μ. Tables 1 and 2 show that μ increases with the 6^{th} power of alamethicin concentration approximately independent of temperature but follows a voltage dependence according to only 2 to 3 elementary charges. On the basis of the simplest kinetic model an aggregate of six alamethicin molecules seems to build up prior to or during pore formation. However, if we assume that the ρ relation reflects an intrinsic property of the alamethicin molecule (a more detailed discussion is given in the last section) actually only two to three molecules seem to be involved in pore formation. We therefore assume an inactive preaggregate of six alamethicin molecules to be situated at the membrane interface out of which only two to three molecules are simultaneously inserted into the membrane by the voltage to create the active pore nucleus.

In contrast to the nearly temperature-independent value of $E_A[\tau_s]$ the formal activation energy $E_A[\mu]$ exhibits a temperature dependence. According to Table 4 at 40 mV under consideration of the temperature independence of $\bar{A}$, $E_A[\mu] \approx 36$ kcal/mol between 18 and 11 °C and $E_A[\mu] \approx 19$ kcal/mol between 32 and 25 °C is calculated.

Effect of Changing Salt Concentration

In the following we consider the effect of changing salt concentration on the experimentally determined parameters. Table 3 shows that the characterizing voltages of λ_∞ and τ_s shift to higher values by lowering salt concentration. In order to compare the systems at identical pore concentrations N_p and identical pore state distributions p_v we consider the

dependence of $\bar{\Lambda}$ on salt concentration. The data of Table 3 show that $\alpha[\tau_s]$ does not change significantly. This implies that the p_v distributions remain approximately constant. On the other hand, the single pore state conductances Λ_v change with changing salt concentration according to $\Lambda_v \propto (C_{salt})^{0.73}$ (Eisenberg *et al.*, 1973). About the same exponential factor $\varepsilon[\bar{\Lambda}] \approx 0.8$ has been obtained from the salt concentration dependence of $\bar{\Lambda}$ (at constant λ_∞) from autocorrelation analysis (Kolb & Boheim, 1977). We now define a parameter $\varepsilon[N_p]$ by $\varepsilon[N_p] = \varepsilon[\lambda_\infty] - \varepsilon[\bar{\Lambda}]$ which describes the dependence of a characteristic voltage at $N_p = \text{const.}$ on salt concentration and equivalently the dependence of the pore concentration N_p at $V = \text{const.}$ on salt concentration. We have shown in the previous section that conditions of $\lambda_\infty = \text{const.}$ correspond to those of $N_p = \text{const.}$ if the alamethicin concentration is varied at a given salt concentration. This means $\delta[\lambda_\infty] = \delta[N_p]$. Table 3 indicates that the ratios $\delta[N_p]/\varepsilon[N_p]$ and $\delta[\tau_s]/\varepsilon[\tau_s]$ are approximately equal. The relative shifts of N_p and τ_s are consequently comparable whether the alamethicin or the salt concentration is changed. This result is consistent with the observation of Gordon and Haydon (1975) that the amount of alamethicin adsorbed at the lipid interface is reduced by lowering salt concentration (salting-out effect). Only to a minor extent τ_s seems to be directly influenced by the ionic strength in the considered salt concentration range.

A difference in salt concentration by a factor of 2 caused a shift of about 10 mV in V_{100} (at 25 °C). As a consequence of the high power dependence on alamethicin concentration this corresponds to a change of about 20% in the interfacial alamethicin concentration. At this point we want to draw attention to the estimated experimental scatter of the λ_∞ values by a factor of 10 mentioned in the Results section. This scatter also is equivalent to a 20% difference in alamethicin concentration. Thus the accuracy of $\pm 10\%$ in reproducing the interfacial alamethicin concentration at a given aqueous alamethicin concentration is about the same as observed with valinomycin (G. Stark, *personal communication*).

Effect of Membrane-Pretreatment

A further point which has to be discussed is the influence of voltage-pretreatment on the properties of the alamethicin-modified lipid membrane. V_{100} and $\alpha[\lambda_\infty]$ change with the extent of membrane preconditioning. At 25 °C, 1 M KCl and 2.5×10^{-7} g/ml alamethicin $V_{100} = 48$ mV and $\alpha[\lambda_\infty] = 6.9$ is found under conditions of zero voltage-pretreatment,

$V_{100}=47$ mV and $\alpha[\lambda_\infty]=6.3$ under conditions of low λ_Φ-pretreatment and $V_{100}=28$ mV and $\alpha[\lambda_\infty]=5.3$ under conditions of high λ_Φ-pretreatment. In the latter case the weakly voltage-dependent conductance is about $\lambda_\Phi(10\text{ mV})\approx 100\ \mu\text{S cm}^{-2}$. For comparison, therefore, V_{100} was obtained by extrapolation from the voltage-dependent part of the I/V curve.

A similar shift to lower voltages with the extent of preconditioning is observed for τ_s. A possible explanation of the preconditioning effect could be a change in the interfacial alamethicin concentration due to a slow reaction, even if the exchange of alamethicin between water and membrane interface is fast. Then λ_∞ and τ_s should shift in parallel corresponding to their power dependence on alamethicin concentration. The experimental data are consistent with this interpretation at 11 °C, but at 25 °C the τ_s values obtained under conditions of high λ_Φ-pretreatment are still about a factor of 3 smaller than the values measured under conditions of low λ_Φ-pretreatment. As we mentioned earlier, the comparison of the τ_v values obtained under single-pore conditions with the τ_f data from multi-pore experiments shows τ_f to be smaller by a factor of 4. As pointed out by Gordon and Haydon (1976) the varying membrane fluidity of different lipids may cause differences of two orders of magnitude in the rate constants of single-pore state transitions. Possibly the membrane became more fluid by the formation of many pores and the occurrence of the weakly voltage-dependent conductance in the above-considered case.

Two additional effects appear under conditions of high λ_Φ-pretreatment. First, the weakly voltage-dependent conductance λ_Φ increases concomitantly to the decrease of V_{100}. Second, a saturation behavior is seen in the I/V characteristic starting at 3–5 mS cm^{-2} (Fig. 5*a*) which could not be observed up to conductances of 10 mS cm^{-2} under conditions of zero voltage- and low λ_Φ-pretreatment.

The time scale of the appearance of λ_Φ lies within minutes to hours. Such slow processes have been observed after alamethicin dissolution in water-ethanol mixtures by measuring the α-helical content (Jung *et al.*, 1975). A more detailed investigation of the weakly voltage-dependent conductance λ_Φ has been carried out by autocorrelation analysis (Kolb & Boheim, 1977). The results of this analysis and of the suzukacillin relaxation experiments (Boheim *et al.*, 1976) suggest that λ_Φ results from pore structures which are converted into the pores of the voltage-dependent conductance $(\lambda_\infty-\lambda_\Phi)$ through mere uptake of additional alamethicin monomers by the pore. A systematical investigation of the relation between λ_Φ and the shift in V_{100} has not yet been done.

The saturation behavior of λ_∞ could be caused by a limited number of alamethicin monomers adsorbed at the membrane interface, by a limited number of the preaggregates at the interface or by a change of the voltage dependence of the p_v distributions due to a limited size of the pore. The time course of the current relaxation followed an inactivation curve, i.e., it passed through a maximum within the saturation range. The time of minutes for this effect is of comparable order to the time for the appearance of the λ_Φ conductance. Fig. 6*a* shows that τ_s bends to the voltage-axis in correspondence to λ_∞ (Fig. 5*a*) whereas μ in Fig. 6*b* still depends exponentially on voltage. This means that τ_s is the saturating quantity.

The first explanation (monomer depletion) mentioned above seems to be quite unlikely since Gordon and Haydon (1975) estimated an interfacial monomer concentration on the order of 10^{12} molecules per cm^2, whereas saturation starts at pore densities of 10^4 cm^{-2}. A limited number of preaggregates would alter Eq. (A.5) to

$$\frac{dN_p}{dt} = k_{m0}(N_m - N_p) - k_{0m} p_0 N_p = k_{m0} N_m - (k_{m0} + k_{0m} p_0) N_p \qquad (22)$$

with N_m: concentration of interfacial preaggregates (mol cm^{-2}) and k_{m0}: rate constant of pore nucleus formation. Since Eq. (22) does not describe the inactivation behavior, N_m could only be reduced in a separate slow reaction. But as can be seen from Fig. 6*b* the product $\lambda_\infty \cdot \frac{1}{\tau_s} = \mu \bar{A}$ still increases with increasing voltage after prolonged preconditioning. Because $\bar{A}$ nearly remains constant it follows that μ increases. This fact seems to rule out the possibility of preaggregate exhaustion.

The third possibility, a restriction in size of the alamethicin pores, implies that the inactivation is caused by an overshoot in the p_v distributions. After a period of seconds a redistribution of the pore subunits should occur. The nature of this redistribution process might be the growth of newly formed pores through the dissociation of monomers from large pores (Baumann & Mueller, 1974; Boheim *et al.*, 1976) or the transfer of monomers which are released from the pore during a pore state transition across the membrane. This mechanism of inactivation has been made likely for monazomycin by Heyer, Muller and Finkelstein (1976). In any case, the number of pore subunits has to be restricted which is difficult to understand on the basis of the results of Gordon and Haydon (1975) that the monomers are 10^6 times in excess. This would only be consistent if only a small fraction of alamethicin monomers having a special con-

formation would be able to form pores. In addition, this would mean that the pores no longer fluctuate independently in the range of λ_∞, τ_s saturation (Kolb & Boheim, 1977).

Preaggregate and Pore Nucleus Formation

It has been proposed by Eisenberg *et al.* (1973), Gordon and Haydon (1975), Hall (1975) and Mueller (1976*a*) that an inactive preaggregate exists at the membrane interface prior to pore formation. On the basis of data of Chelack and Petkau (1973), Mueller (1975*a*) assumed an interfacial alamethicin concentration of 10^7 molecules per cm^2 at an aqueous alamethicin concentration of 10^{-7} M. On the other hand, Gordon and Haydon (1975) calculated from measurements of alamethicin adsorption onto lipid monolayers an interfacial concentration of about 10^{12} molecules per cm^2 for the same antibiotic concentration. In addition they obtained evidence that a large excess of monomers over aggregates exists at the membrane interface. From the value of Gordon and Haydon (1975) the mean distance between two monomers is calculated to be about 10 nm. With a lateral diffusion coefficient of 10^{-8} cm^2 sec^{-1} this distance corresponds to a diffusion time of about 100 μsec which would be fast enough to account for the observed pore-state transitions.

The reaction of the active pore with alamethicin monomers was introduced as a hypothesis into the kinetic equations by Boheim (1974) and Baumann and Mueller (1974). This hypothesis is supported by the arguments presented in this paper. We therefore substitute in Eqs. (A.1) and (A.7)

$$\begin{aligned} k^*_{\nu-1,\nu} &= k_{\nu-1,\nu} \cdot N \\ K^*_\nu &= K_\nu \cdot N. \end{aligned} \tag{23}$$

N turned out to be a constant quantity during pore-state fluctuations (Boheim, 1974). But up to now it has not been demonstrated whether N is the concentration of monomers inserted anywhere into the membrane or if a monomer can only be inserted into the membrane during the uptake by the pore.

The dependence of the pore formation rate on voltage and alamethicin concentration may give some insight into the pore nucleation mechanism. As already mentioned, the alamethicin concentration dependence of the pore formation rate μ gives evidence that at the membrane interface a preaggregate of 6 molecules is formed. A possible configuration of the

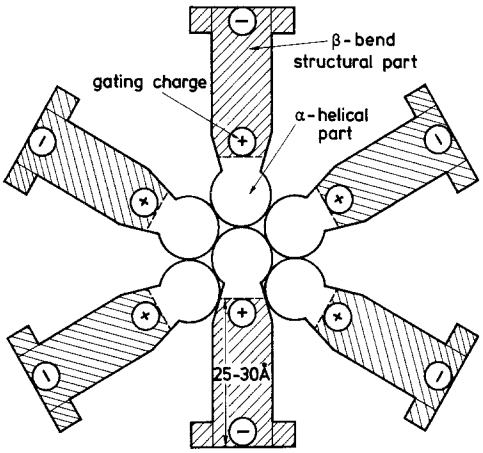

Fig. 7. Proposed hexameric configuration of the interfacial alamethicin aggregate (micelle) which is thought to be the preaggregate from which pore formation starts. The α-helical parts arrange together as a consequence of hydrophobic interactions and may be oriented toward the membrane interior, whereas the flexible (β-bend) molecule parts are stretched out at the membrane interface. In case of pore nucleation two (or even three) molecules are simultaneously inserted into the membrane each transferring an equivalence of a positive gating charge across the membrane. Then aggregation of the β-bend parts occurs by forming the nonconducting dimer (or the conducting trimer) pore. The alamethicin molecule is branched at the C-terminal end into a phenylalaninol and a negatively charged glutamine. It is likely that two hydrogen bonds between adjacent molecules may be formed at these groups. After pore nucleation the peculiar hexameric preaggregate structure has disappeared

interfacial preaggregate is proposed in Fig. 7. The α-helical parts of the alamethicin molecules arrange together as a consequence of hydrophobic interactions and may be oriented toward the membrane interior, whereas the flexible (β-bend) parts are stretched out at the membrane interface. In the peculiar structure shown in Fig. 7 the hexameric compact arrangement is assumed to be favored although smaller and larger aggregates are also feasible. The hypothesis of Gordon and Haydon (1975) that alamethicin pores could be switched on by voltage pulses of either polarity was not taken into consideration, since this has not been proven for multipore systems.

Now, if we assume that the magnitude ρ [Eq.(17)] describing the voltage-alamethicin concentration relation reflects an intrinsic property of a single alamethicin molecule, the number of molecules out of the hexameric preaggregate which are actually moved by the voltage during the pore nucleation process can be estimated from $\alpha[\mu]$. Under the assumption that the forward rate constant of pore nucleation k_{m0} is approximately independent of voltage and alamethicin concentration we

write

$$\alpha[\mu] = \rho \cdot \delta^*[\mu], \tag{24}$$

whereby $\delta^*[\mu]$ is the mean number of monomers involved in single-pore nucleation. Values between $\delta^*[\mu] = 3.0$ at 25 °C and $\delta^*[\mu] = 2.4$ at 4 °C are obtained. Consequently, we propose that two or three molecules are simultaneously inserted into the membrane to form the pore nucleus. The validity of the ρ relation would make it unlikely that the preaggregate as a whole is inserted into the membrane. This relation, for which we have given evidence in case of the p_v distributions and the slow relaxation time and which was proposed to reflect an intrinsic property of one alamethicin molecule, is assumed to be transferable to the pore nucleation process, too. Thus on the basis of the value of ρ at low temperature a dimer nucleus seems to be favored whereas at 25 °C the nucleus may be a trimer. Structural considerations of Boheim (1974) which are not influenced by the recently proposed linear structure of alamethicin (Martin & Williams, 1975) had led to the proposition of a dimer as the lowest nonconducting pore state at low temperature. Then the trimer would be the first conducting state. These considerations are consistent with the multi-pore results in that the dimer seems to be the pore nucleation state at low temperature. Eisenberg *et al.* (1973), who measured at room temperature, did not observe a nonconducting porestate which seems to indicate a trimer as nucleation state in that case.

The alamethicin molecule is probably branched at the C-terminal end into a phenylalaninol and a negatively charged glutamine (Martin & Williams, 1975, 1976; Jung *et al.*, 1976). The formation of two hydrogen bonds between adjacent molecules may only be possible at these groups (Boheim *et al.*, 1976). For the activation energy of the mean life-times of the pore-states we measured $E_A[\tau_v] = 12$ kcal/mol which is comparable to the activation energy of the corresponding rate constants measured by Gordon and Haydon (1976) of $E_A[k_{v,v\pm1}] \approx 10$ kcal/mol. These values are consistent with the formation (decay) of two hydrogen bonds during the uptake (release) of one alamethicin molecule by the pore.

In the following we summarize the preaggregate hypothesis. After membrane-formation alamethicin molecules diffuse to the membrane interface and become adsorbed onto it. Through the contact with the hydrophobic membrane interior the mean length of the α-helix of an alamethicin monomer, which amounts to about 20% of the molecule in water, increases in a very slow reaction up to 40% (Jung *et al.*, 1975). With the increase of the α-helix the tendency of the monomers to form

preaggregates increases. A voltage which is applied to the system first acts on the α-helical part of the molecule. The α-helix gives an intrinsic dipole moment with a component perpendicular to the membrane interface, the positive (N-terminal) side pointing to the opposite interface. Surface potential studies of alamethicin monolayers at the air/water interface (Gordon & Haydon, 1975) showed that the normal component of the surface dipole changes by ca. $2 \cdot 10^{-29}$ C m for each polypeptide molecule. An α-helix of nearly two turns with three hydrogen bonds would account for this value which is consistent with the proposed structure of alamethicin. Now, this dipole moment may provide the trigger function for monomer insertion and pore nucleation, respectively. The α-helix may be pushed into the membrane interior by the inhomogeneous electric field at the membrane interface. It is evident that the trigger effectivity of the monomer depends on the size of its α-helical part which is subject to a slow change in equilibrium distribution as mentioned above. The experimentally observed larger voltage-dependent change in the Gibb's free energy may somehow be associated with the orientation of the β-bend structural part from the horizontal at the membrane interface perpendicular to it, thus bridging the membrane. A stable pore nucleus is only created if two or three molecules are simultaneously inserted into the membrane. It then starts to grow by the uptake of additional monomers. After pore nucleation the peculiar hexameric preaggregate structure has disappeared so that nonintegrated monomers may diffuse away. Thus the mean life-time of a pore is not only determined by its mean state which has been adopted according to the equilibrium distribution with the large pool of interfacial monomers, but also by the dissociation rate of the nonintegrated monomers from the nucleus. If dissociation occurs before an equilibrium state with the monomer pool is established, the pore nucleus decays according to this second faster decay mechanism.

The strong decrease of the formal activation energy of the pore formation rate, $E_A[\mu]$, with increasing temperature may be explained by a reduced tendency of monomers to aggregate. This would cause a decrease in the preaggregate concentration at higher temperatures. The reduction in the mean pore state with increasing temperature also seems consistent with this interpretation.

Conclusion

In the following we state the main results of the detailed analysis of the alamethicin multi-pore system which is presented in this paper.

1. The behavior of the alamethicin system depends quantitatively and to some extent also qualitatively on its voltage-pretreatment. Extensive voltage-pretreatment leads to a forced appearance of the weakly voltage dependent conductance λ_Φ and to a saturation behavior at high conductances.

2. A detailed study of the two relaxation times found with the alamethicin system is carried out. Activation energies of $E_A[\tau_s] = 26\text{–}34\,\text{kcal mol}^{-1} = 109\text{–}142\,\text{kJ mol}^{-1}$ for the slow relaxation time τ_s and $E_A[\tau_f] = 12\,\text{kcal mol}^{-1} = 50\,\text{kJ mol}^{-1}$ for the fast relaxation time τ_f, which is the same as $E_A[\tau_v] = 12\,\text{kcal mol}^{-1}$ for the mean life-time τ_v of the most probable pore states v, are obtained.

3. A fundamental relation [Eq. (A.6)] between single-pore and multipore properties of the alamethicin system is derived from the phenomenological behavior of the alamethicin pore under the experimentally verified assumptions that the pore states are adopted in consecutive order and that the pore only disappears from its lowest (first adopted) state. Since only these general properties are taken into account by the mathematical treatment, Eq. (A.6) is applicable to each of the molecular models of alamethicin action proposed up to now.

4. On the basis of functional analytical arguments a relation [Eq. (17)] between the voltage and alamethicin concentration dependence of τ_s is derived from Eq. (A.6) which leads to the conclusion that a single alamethicin molecule associated with the equivalence of about one positive elementary charge is taken up or released by the pore during each pore state transition. This result is consistent with a molecular model in which interfacial monomers are in equilibrium with the fluctuating pore, e.g. the "barrel stave" model. However, molecular models are excluded, where the different pore conductance states are created by conformational changes of an aggregate composed of a fixed number of molecules.

5. The pore state distributions p_v change with voltage, i.e. higher pore states become more probable at higher voltages. This result is concluded from the voltage dependence of the mean pore conductance $\bar{\Lambda}$ and from that of the slow relaxation time τ_s. It is consistent with the results obtained from single-pore analysis by Boheim (1974).

6. The pore state distributions p_v change with temperature. Lower temperature leads to higher pore states and consequently to a strong increase in the mean life-time of a fluctuating pore.

7. An estimate of the mean pore state $\bar{v}$ is obtained from the parameter $\alpha[\tau_s]$ which describes the voltage dependence of τ_s.

8. Changing salt concentration or alamethicin concentration results in the same effects. This is consistent with the experimental finding of Gordon and Haydon (1975) that the adsorption equilibrium of alamethicin at the membrane interface is ionic strength dependent (salting-out effect).

9. Some evidence is given for the existence of a preaggregate consisting of six alamethicin molecules at the membrane interface. The pore nucleus is formed out of this preaggregate by simultaneous insertion of two or three molecules into the membrane after voltage application.

Appendix

Derivation of the Voltage-Alamethicin Concentration Relation ρ for τ_s [Eq. (17)]

In the following we present the basic equations as phenomenological descriptions of the experimental observations *without reference to a particular molecular model.* Emphasis is put on the direct correlation between single-pore and multi-pore phenomena.

On the basis of single-pore experiments all intrapore reactions can be described by first-order differential equations (Baumann & Mueller, 1974; Boheim, 1974; Gordon & Haydon, 1976; Mueller, 1976*a*).

$$\frac{dN_\nu}{dt} = k^*_{\nu-1,\nu} \cdot N_{\nu-1} - k_{\nu,\nu-1} N_\nu - k^*_{\nu,\nu+1} N_\nu + k_{\nu+1,\nu} N_{\nu+1}, \qquad \nu = 1, 2, 3 \ldots \text{[3]} \tag{A.1}$$

N_ν: concentration of pores (mol cm^{-2}) in state ν; $k^*_{\nu-1,\nu}$ and $k_{\nu,\nu-1}$: rate constants of forward and backward reaction, respectively. $\nu = 1$ is the first conducting state. The index 0 is given to a nonconducting state which had to be postulated from the statistical analysis of single-pore fluctuations (Boheim, 1974).

In the case of multi-pore experiments the slow relaxation process has been shown to be of first order. We write (Eisenberg *et al.*, 1973; Boheim *et al.*, 1976)

$$\frac{dN_p}{dt} = \mu - k_b N_p \tag{A.2}$$

3 With $k^*_{\nu-1,\nu} = k_{\nu-1,\nu} \cdot N$, Eq. (A.1) becomes identical with Eq. (7) of Boheim (1974). Eq. (A.1) also agrees formally with the mathematical description of the pore state transitions given by Gordon and Haydon (1976).

with μ: pore formation rate (mol cm^{-2} sec^{-1}) and k_b: pore decay rate (sec^{-1}).

As outlined in the Discussion, the pore formation rate μ does not show saturation behavior even at its highest values. Therefore, it seems to be reasonable to assume that μ remains constant during the relaxation process.

From the fluctuation records it is seen that a burst mostly starts at the lowest conductance level and disappears from that level (Eisenberg *et al.*, 1973; Boheim, 1974; Gordon & Haydon, 1975, 1976; Boheim *et al.*, 1976). This observation is consistent with the notion that the active pore is created by a nucleation process in state $v=0$ and that the occurrence of higher pore states $v \geqq 1$ is the consequence of growth or reforming processes of this nucleus which are described by Eq. (A.1). This leads to the following equation for N_0, the concentration of pores in the lowest state

$$\frac{dN_0}{dt} = \mu - k_{0m} N_0 - k_{01}^* N_0 + k_{10} N_1, \tag{A.3}$$

k_{0m}: rate constant of decay of state 0.

With $N_p = \sum_{v=0}^{\infty} N_v$ and Eq. (A.1) we obtain

$$\frac{dN_p}{dt} = \sum_{v=0}^{\infty} \frac{dN_v}{dt} = \mu - k_{0m} N_0. \tag{A.4}$$

Because $p_v = N_v/N_p$, $v=0, 1, 2, \ldots$ is the probability to find a pore in state v, the relation

$$\frac{dN_p}{dt} = \mu - k_{0m} p_0 \cdot N_p \tag{A.5}$$

holds. Thus we obtain with respect to the multi-pore system (Eq. A.2)

$$\frac{1}{\tau_s} = k_b = k_{0m} p_0. \tag{A.6}$$

It has been shown that the equilibrium constants K_v^* between neighboring pore states may be expressed by [Eq. (25) in Boheim, 1974]

$$\frac{p_v}{p_{v-1}} = \frac{N_v}{N_{v-1}} = K_v^* = \Gamma \cdot \exp\left\{\frac{\alpha F V - v \cdot \Delta G_R}{RT}\right\} \tag{A.7}$$

with Γ: proportionality constant; αFV: voltage dependent part of the change in Gibb's free energy per mol associated with pore state transitions;

$\nu \cdot \Delta G_R$: voltage independent part of the change in Gibb's free energy per mol associated with pore state transitions.

The dependence of the proportionality constant $\Gamma(N_s)$ on the interfacial alamethicin concentration N_s has not been measured experimentally. $\Gamma(N_s)$ was introduced by Boheim (1974) as a hypothesis, since the observed constancy of Γ, α and ΔG_R in different pore states, i.e. independent of ν, seemed to indicate that a definite subunit was involved in each pore state transition.

The zero state distribution p_0 is given by

$$p_0 = \frac{N_0}{N_p} = \frac{N_0}{N_0 + K_1^* N_0 + K_2^* K_1^* N_0 + \cdots} = \frac{1}{1 + \sum_{\nu=1}^{\infty} \prod_{i=1}^{\nu} K_i^*}. \tag{A.8}$$

Introducing Eq. (A.7) one obtains

$$p_0 = \frac{1}{1 + \sum_{\nu=1}^{\infty} \left(\Gamma \cdot \exp\left\{\alpha \frac{FV}{RT}\right\}\right)^{\nu} \cdot \exp\left\{-(\nu+1) \cdot \frac{\nu}{2} \frac{\Delta G_R}{RT}\right\}}. \tag{A.9}$$

Thus the dependence of p_0 on Γ and the voltage may be written as

$$1/p_0 = f\left(\Gamma \cdot \exp\left\{\alpha \frac{FV}{RT}\right\}\right). \tag{A.10}$$

Now, Figs. 8*a* and *b* demonstrate that the sum of terms with different exponential factors in Eq. (A.9) is described within experimental accuracy by only one exponential term for an interval of about 40 mV with respect to the voltage dependence and for a change of about a factor of 5 with respect to the dependence on Γ. This range of suitable approximation of $1/p_0$ by a single term, whereby the exponential parameters $\alpha[1/p_0]$ and $\delta[1/p_0]$ in Eq. (A.11) are not necessarily integral numbers, is the same which was experimentally accessible in the case of the determination of τ_s.

In Fig. 8*a* Γ was held constant and the voltage V was changed, whereas in Fig. 8*b* V was fixed and Γ varied. Analogous to Eqs. (1) and (2) we find

$$\frac{1}{p_0} \propto (\Gamma)^{\delta[1/p_0]} \cdot \exp\left\{\alpha[1/p_0] \cdot \frac{FV}{RT}\right\}. \tag{A.11}$$

The comparison of Eqs. (A.10) and (A.11) shows that

$$1/p_0 = f\left(\Gamma \cdot \exp\left\{\alpha \frac{FV}{RT}\right\}\right) \propto \left(\Gamma \cdot \exp\left\{\alpha \frac{FV}{RT}\right\}\right)^{\alpha[1/p_0]} \tag{A.12}$$

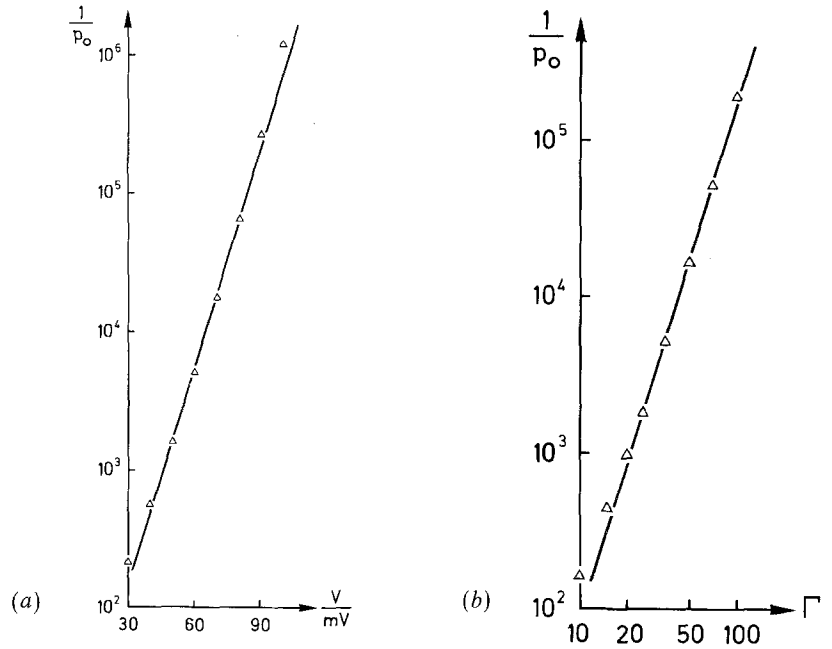

Fig. 8. Reciprocal $1/p_0$ of the zero pore state probability as function of membrane voltage (*a*) and parameter Γ (*b*) according to Eq. (A.9). The values of the parameters $\alpha = 0.93$ and $\Delta G_R = 0.91$ kcal/mol have been taken from the statistical analysis of pore state fluctuations with di-(22:1)-lecithin membranes at 11 °C (Boheim, 1974). In (*a*) $\Gamma = 35$ was chosen to account for the given p_0 data set of this analysis. In (*b*) $V = 60$ mV was held constant whereas Γ varied. In both cases within an interval of 40 mV and a change of a factor of 5 in Γ, respectively, the calculated points drawn in half-logarithmic plots fit into a straight line approximation

with $\alpha[1/p_0] = \alpha \cdot a[1/p_0]$; $\delta[1/p_0] = a[1/p_0]$ is valid, whereby the relation $\alpha[1/p_0]/\delta[1/p_0] = \alpha$ holds.

The aqueous alamethicin concentration and voltage dependence of the slow relaxation time τ_s (*see* Table 1) has been determined according to Eqs. (1) and (2) in terms of

$$\tau_s \propto (C_{AL})^{\delta[\tau_s]} \cdot \exp\left\{\alpha[\tau_s] \cdot \frac{FV}{RT}\right\}. \tag{A.13}$$

As a consequence of Eq. (A.6), Eq. (A.13) may be compared with Eq. (A.12) taking into account the following considerations:

1. We assume that the interfacial concentration N_s of alamethicin monomers is proportional to the aqueous concentration C_{AL} at a given salt concentration within the experimentally considered alamethicin concentration range:

$$N_s = \gamma_{AL} \cdot C_{AL}. \tag{A.14}$$

2. We assume that α and ΔG_R are independent of N_s and that Γ depends with the x^{th}-power on N_s

$$\Gamma \propto (N_s)^x \propto (C_{AL})^x. \tag{A.15}$$

3. The rate constants $k_{v,\,v-1}$ show only a weak voltage dependence of varying sign in the case of lecithin membranes (Boheim, 1974; Mueller, 1976*a*). In extension of this fact we assume the nucleus decay rate $k_{0\,m}$ to be approximately independent of voltage and alamethicin concentration.

By comparison of the exponential parameters of Eqs. (A.12) and (A.13) under consideration of Eq. (A.15) we obtain the relations

$$\alpha[\tau_s] = \alpha \cdot a\left[\frac{1}{p_0}\right]; \quad \delta[\tau_s] = x \cdot a\left[\frac{1}{p_0}\right]. \tag{A.16}$$

For description of the relation between voltage and alamethicin concentration dependence of τ_s we define a factor ρ by

$$\rho = \frac{\alpha}{x}. \tag{A.17}$$

Note Added in Proof: In a recently published abstract, R. E. Yantorno, S. Takashima and P. Mueller (*Biophys. J.* **17**:87a, 1977) reported about the intrinsic dipole moment of the alamethicin molecule. They observed a value of about 67 Debye (2.2×10^{-28} C m) which corresponds to two opposite charges separated by a distance of 14 Å (solvent: 25% ethanol in dioxane). Yantorno *et al.* assigned this dipole moment primarily to the α-helical part of the molecule.

The orientation of such a dipole moment in an electric field would account for only about 50% of the voltage dependence which was expected to originate from a single alamethicin molecule by Boheim (1974) and in this paper, respectively. The equilibrium constant K_v^* [Eq. (A.7)] would be given by

$$K_v^* = K_v' \cdot \exp\left\{\frac{\mu E}{kT}\right\} = K_v' \cdot \exp\left\{\frac{\mu}{ed} \cdot \frac{FV}{RT}\right\}$$

with μ: dipole moment of a single alamethicin molecule, E: electric field strength, μE: voltage-dependent part of the difference in Gibb's free energy between two neighboring pore states, k: Boltzmann constant, e: elementary charge, d: length of the alamethicin pore. The parameter α introduced by Boheim (1974) is identical to $\alpha = \mu/ed$. With $\mu = 67$ Debye from Yantorno *et al.* (1977) and d = 30 Å, which is a reasonable length of the alamethicin pore (*see* Fig. 7), one obtains $\alpha = 0.46$. However, this is only about half the value obtained experimentally by Boheim (1974) from single pore analysis with lecithin membranes ($\alpha = 0.93$). As discussed in this paper, the additional voltage-dependent change in Gibb's free energy is proposed to result from the orientation of the β-bend structural part of the molecule. This peculiar conformation may not be adopted in a homogeneous solvent but at a lipid-water interface.

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