DIPOLE MOMENT OF ALAMETHICIN AS RELATED TO VOLTAGE-DEPENDENT CONDUCTANCE IN LIPID BILAYERS

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ABSTRACT The dipole moment of alamethicin, which produces voltage-dependent conductance in lipid-bilayer membranes, was measured in mixed solvents of ethanol and dioxane. The value of the dipole moment was found to increase from 40 to 75 DU (Debye units), as the concentration of ethanol increased from 0 (pure dioxane) to 40%. The relaxation frequency of alamethicin also changes from 10 to 40 MHz, depending upon the concentration of ethanol in mixed solvents. The length of alamethicin was calculated by using the relaxation time and was found to range from ~40 to 20 Å. The dipole moment was independently calculated from voltage-dependent conductance and compared with the measured value. The calculated value was found to be larger than the value of direct measurements, indicating that several alamethicin molecules are required to form a conducting pore and that their dipole moments are oriented parallel to each other.

INTRODUCTION

Voltage-dependent conductance is the very basis of electrical excitability of nerve and muscle membranes. Hodgkin and Huxley (1952) suggested that one of the possible mechanisms that could be responsible for the voltage-dependent conductance of nerve might be the rotation of dipolar particles in ionic channels. Since then, numerous models and theories of electrical excitability have been developed based on similar concepts. (For a summary of these theories, see Yantorno, 1978.) Observation of gating current (Armstrong and Bezanilla, 1973; Keynes and Rojas, 1974) and voltage-dependent capacitance of nerve membranes (Takashima and Yantorno, 1975) seems to provide experimental support for the dipole theory of nerve excitability.

With the introduction of lipid bilayers (Mueller and Rudin, 1963), it has been possible, in recent years, to simulate biological cell membranes. It has also been possible with the use of a peptide (alamethicin) to produce voltage-dependent conductance in these bilayers (Mueller and Rudin, 1968*a*). In addition, alamethicin was reported to produce action potentials that are similar to those found in natural systems (Mueller and Rudin, 1968*b*). Baumann and Mueller (1974) suggested that the dipole moment of alamethicin may be responsible for this voltage-dependent conductance. Subsequently, Hladky et al. (1974), Haydon (1975), and Gordon and Haydon (1975) postulated that the dipole moment may be on the order of 16–26 DU (Debye units, 10^{-18} esu cm; esu, electrostatic units).

It was our desire to find out whether alamethicin indeed possessed a dipole moment, and then to determine its magnitude to ascertain whether the value would be large enough to account for the observed voltage-dependent conductance of lipid bilayers doped with alamethicin.

MATERIALS AND METHODS

Materials used were spectro-grade *p*-dioxane (Matheson Co., Inc., East Rutherford, NJ) and ethyl alcohol, analytical grade (Publicker Industries, Inc., Philadelphia, PA). Alamethicin was a gift from Dr. G. B. Whitfield, Jr. of the Upjohn Co., Kalamazoo, MI, and was used as received. Mixtures of ethanol and dioxane were used as solvents. All references to their concentrations are based on percent by volume.

A Shering bridge, Hewlett-Packard-Boonton RX 250B meter was used for the dielectric measurements. Its frequency range is 0.5–250 MHz. With the use of a Wayne-Kerr bridge model B221 (200 Hz to 20 kHz), it was possible to extend the resistance range of the RX meter to 1 MΩ and increase the resolution of resistance reading between 50 and 100 kΩ. The capacitance range of the RX meter is 0–23 ± 0.01 pF. The capacitance range was increased by zeroing the meter in the inductance (negative capacitance on the meter) range, thereby providing an increase of 6 pF to the total capacitance. The dielectric measurement cell was constructed of brass, and all surfaces that were in contact with the solutions were gold-plated. Temperature control of the cell was obtained

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from a copper coil soldered to the outside of the cell jacket and connected to a circulating heating and cooling unit (Model 2095, Forma Scientific, Inc., Marietta, OH). The temperature range was from 0° to 70° ± 0.02°C. For the viscosity measurements of mixed solvents, an Ostwald-Cannon-Fenske kinematic viscometer (size 150) was used.

The dielectric cell is a modified concentric cylinder. Eq. 1 was used to calculate the value of the dielectric constant of the material in the cell, i.e.,

$$C = \epsilon C_0 + C_s \qquad (1)$$

where C is measured cell capacitance with a sample, C_0 is the capacitance of the empty cell, C_s is stray capacitance, and ϵ is the relative dielectric constant of the material.

Two center electrodes with different sizes were used, and their respective cell constants (C_0) and stray capacitances (C_s) were 3.35 ± 0.012 pF and 2.04 ± 0.008 pF for electrode 1, and 3.235 ± 0.033 pF and 2.961 ± 0.022 pF for electrode 2. The method for inductance compensation at high frequencies was outlined by Schwan (1963), and the correction can be made by use of Eqs. 2 and 3:

$$R_x = R_p(1 + \omega^2 LC_p) + (\omega L/R_p) \qquad (2)$$

$$c_x = \frac{C_p(1 + \omega^2 LC_p) + (\omega/R_p)}{(1 + \omega^2 LC_p)^2 + (\omega L/R_p)^2}, \qquad (3)$$

where C_p and R_p are measured capacitance and resistance, C_x and R_x are corrected capacitance and resistance, L is series inductance, and ω is angular frequency. Series inductance was determined using the following technique. Because the resistance of the sample used in these experiments is very high, the resistive components of Eq. 3 can be eliminated without serious errors. We obtain, therefore, the following equation:

$$C_x = C_p/(1 + \omega^2 LC_p) \qquad (4)$$

or

$$1/C_x - 1/C_p = \omega^2 L. \qquad (5)$$

Because C_p is a measured quantity, ω is known, and C_x can be obtained by extrapolation of $1/C_p$ to low frequencies, it is possible to determine the value of L from the slope of the plot $(1/C_x - 1/C_p)$ vs. ω^2. The determination was repeated for both cells with pure dioxane and the values of L were found to be 1.131 and 1.045×10^{-8} H, respectively.

Because of the difficulties in adequately defining the exact internal field near polar molecules, the dipole moment is best obtained from an empirical equation by Oncley (1943), i.e.,

$$\mu = [9{,}000\, kT \cdot M(\Delta\epsilon/g)]/4\pi hN \quad \text{(esu cm)} \qquad (6)$$

where $\Delta\epsilon/g$ is the dielectric increment per gram per liter, M is molecular weight, h is an empirical parameter having a numerical value of 5.8 for amino acids and peptides, and N is Avogadro's number.

The center frequency is usually determined from the midpoint of dispersion curves or from the peak of dielectric loss ϵ''. However, in our experiment, a special method had to be introduced because the dispersion of alamethicin was located at very high frequencies in mixed solvents with a high ethanol content, and our equipment was not capable of establishing the entire dispersion curve. To circumvent this difficulty, the Fuoss-Kirkwood equation (1941) was used:

$$\epsilon'' = \epsilon''_m \sec h[B \ln(f_c/f)], \qquad (7)$$

where ϵ'' is the dielectric loss at a given frequency, ϵ''_m is the maximum dielectric loss at the center frequency, and B is a parameter that has a value of 1 for Debye dispersion (1929). If $\cos h^{-1}(\epsilon''/\epsilon''_m)$ is plotted against $\ln(f_c/f)$, a straight line will be obtained. The center frequency, or relaxation frequency, is found from the frequency intercept of this plot (Fig. 2). Also, the value of B can be obtained from the slope of this plot and is a measure of the width of dispersion.

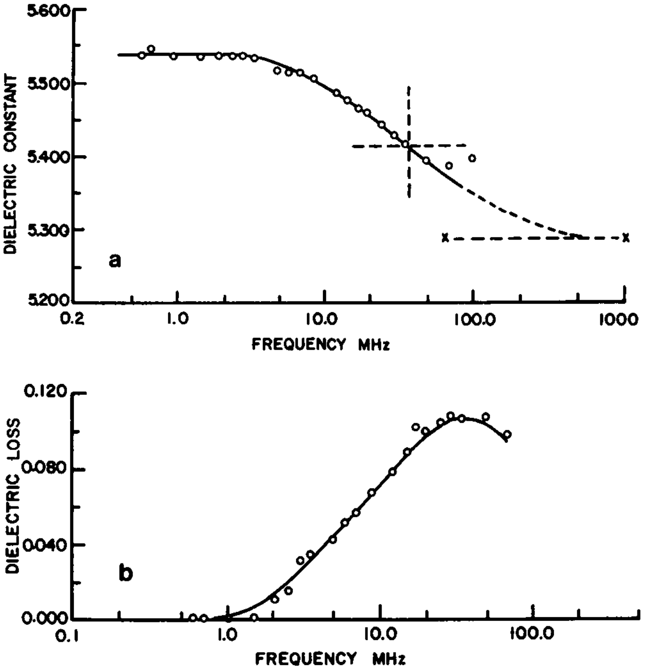

FIGURE 1 (*a*) Dielectric constant vs. frequency. The concentration of alamethicin is 0.75 g/liter (0.38 mM) and the ethanol concentration is 30% (vol/vol). The horizontal line × is a high frequency dielectric constant (ϵ_∞) determined by use of the Fuoss-Kirkwood equation. The cross indicates the center frequency f_c. (*b*) Dielectric loss (ϵ'') vs. frequency. The sample is the same as in *a*. Temperature is 25°C.

RESULTS

One of the dispersion curves of alamethicin obtained in a mixed solvent (30% ethanol and 70% dioxane) is shown in Fig. 1*a*. In this figure, the horizontal line × indicates the dielectric constant at high frequencies (ϵ_∞). As shown, the relaxation frequency is located at a very high frequency in this case, and the Fuoss-Kirkwood plot discussed above had to be used to find ϵ_∞ and the midpoint of the dispersion curve. Fig. 2 shows the plot obtained with the data shown in Fig. 1*b*. From this plot the center frequency f_c of 37.2 MHz and the dispersion parameter $B = 0.80$ were found.

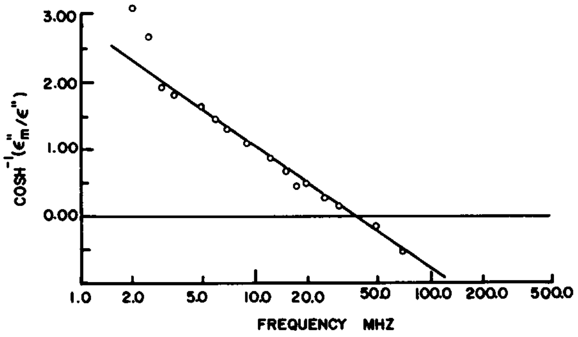

FIGURE 2 Fuoss-Kirkwood plot to determine the center frequency of the dispersion shown in Fig. 1*a*. ϵ'' is the dielectric loss and ϵ''_m is the value of the maximum dielectric loss. From this graph, we obtain values f_c – 37.2 MHz and β – 0.80.

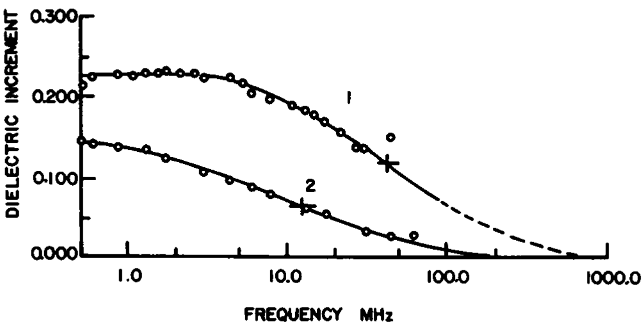

FIGURE 3 Dielectric increments ($\epsilon - \epsilon_\infty$) of alamethicin solutions in mixed solvents. Curve *1* is obtained with 35% (vol/vol) ethanol in dioxane and curve *2* is obtained with 15% (vol/vol) ethanol. The crosses indicate the center frequencies.

This frequency must coincide with the peak frequency of dielectric loss. As shown by Fig. 1*b*, the peak of dielectric loss is found around 40 MHz and is in excellent agreement with the one found using the Fuoss-Kirkwood method.

The measurements of dielectric constant of alamethicin were repeated in mixed solvents with varying ethanol contents, and two of these results are shown in Fig. 3. These curves were obtained with ethanol concentrations of 15 and 35%. As shown in Fig. 3, the increase in the concentration of ethanol is reflected in the dielectric dispersion in three ways: (*a*) increase in the magnitude of the dielectric increment, (*b*) shift in the center frequency to higher regions, and (*c*) narrowing of the dispersion curves (approaching the Debye curve). These changes, particularly the increase in dielectric increment, could be due to aggregation of alamethicin molecules in ethanol. To test for aggregation, the dielectric constant was determined as a function of alamethicin concentrations for two solvents with different alcohol contents (15 and 40%) and are shown in Fig. 4*a* and *b*. In spite of the scattering of measured points, this figure shows the absence of bending of the plots, an indication of molecular association.

In addition, the intermolecular interactions hinder dipolar rotation and give rise to larger relaxation times. In particular, these interactions would be marked at high concentrations and, therefore, the real relaxation frequency or relaxation time must be obtained by extrapolation of the curve to infinite dilution. As shown in Fig. 5, the relaxation time shows a clear trend to bend upward at concentrations >1 g/liter. However, the measured relaxation time approaches a limiting value between 0.5 and 1 g/liter. The value of τ obtained from this figure is 3.3×10^{-9} s.

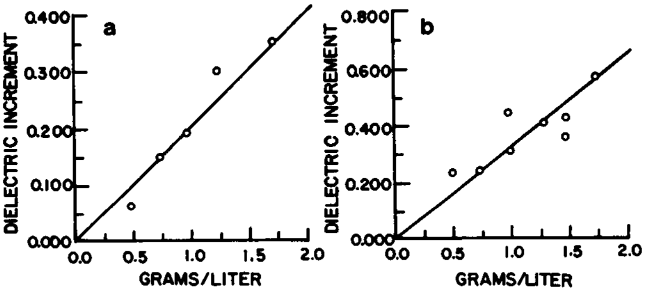

FIGURE 4 The dielectric increments of alamethicin ($\epsilon_0 - \epsilon_\infty$) in two different mixed solvents (15[*a*] and 40%[*b*] ethanol). The linearity of these plots indicates the absence of molecular association.

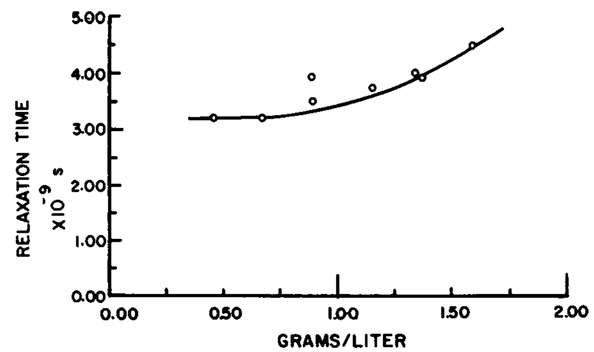

FIGURE 5 Relaxation time of the dispersion as a function of alamethicin concentration. Ethanol concentration is 40% (vol/vol) in dioxane. The upward curve at high concentrations indicates hindered rotation.

The dipole moment of alamethicin was calculated using Eq. 6, and the value of dielectric increment obtained at infinite dilution at various ethanol concentrations. The values of the dipole moment in these solvents are shown in Fig. 6*a*. As shown, the dipole moment increases from a value of 40 DU in pure dioxane to a limiting value of 75 DU.

Relaxation time is usually defined as the time required for the dipolar molecule to undergo a transition from polarized states to random distribution. Therefore, the

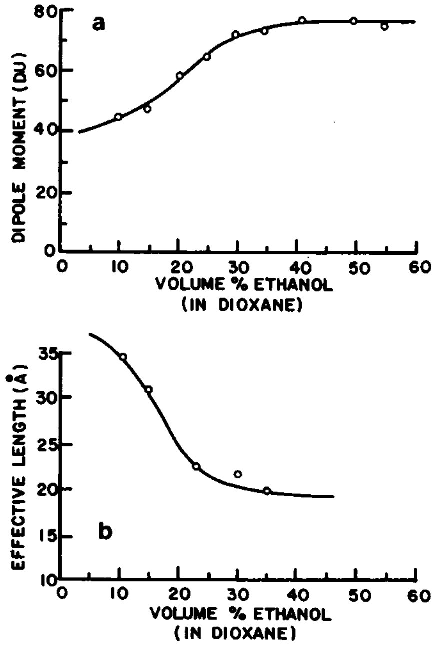

FIGURE 6 (*a*) The dipole moment of alamethicin as a function of the ethanol concentration in dioxane. (*b*) The effective length of alamethicin as a function of the concentration of ethanol in dioxane.

relaxation time is directly related to rotary diffusion and, consequently, to the geometry of the molecule. Debye (1929) derived, based on the Stokes' theorem, an equation that relates relaxation times with the radius of spherical molecules, i.e.,

$$\tau = 4\pi R^3\eta/kT, \tag{8}$$

where R is the radius and η is the viscosity of the solvent. This equation applies only to spherical molecules. Because alamethicin has an elongated conformation (Boheim, 1974), use of Eq. 8 is not appropriate.

If alamethicin is assumed to be a rigid rod, we can use the Perrin equation (1934) to calculate its length instead of the Debye equation.

$$\theta = \frac{3kT}{16\pi\eta a^3}[2\ln(2a/b) - 1], \tag{9}$$

where a and b are major and minor axes of the rod and θ is the rotary diffusion constant and is related to relaxation time by $\tau = 1/2\theta$. To calculate the length of the rod, one has to know an approximate value of the ratio a/b and the viscosity of the solvent η. The viscosities of mixed solvents were determined using a capillary viscometer as mentioned above. The method to determine axial ratios of ellipsoidal molecules using dielectric dispersion curves was detailed by Oncley (1943). The calculation is based on the following assumptions: (*a*) rotary relaxation time is reasonably close to dielectric relaxation time, and (*b*) the molecule is approximated as an ellipsoid of revolution whose rotation is characterized by two time constants. Under these conditions, the dielectric relaxation of the molecules can be defined by two relaxation terms, i.e.,

$$\epsilon' - \epsilon_\infty = \frac{\Delta\epsilon_1}{1 + (\omega\tau_1)^2} + \frac{\Delta\epsilon_2}{1 + (\omega\tau_2)^2}, \tag{10}$$

where $\Delta\epsilon_1$ and $\Delta\epsilon_2$ are dielectric increments along major and minor axes. Likewise, τ_1 and τ_2 are relaxation times along major and minor axes. Both τ_1 and τ_2 can be reduced to a common factor, i.e., the relaxation time of an equivalent sphere (τ_0) and a shape factor. This shape factor can be calculated using the Perrin theory (1934). Using these methods, Oncley calculated a family of theoretical dispersion curves and demonstrated that they can be used to determine the axial ratio of dipolar molecules as long as the angle between the dipole moment and the major molecular axis is known or assumed.

Fig. 7 shows two of these curves for the axial ratio of 9 (curve *1*) and 10 (curve *2*). The dipole angle was assumed to be 30° for curve *1* and 45° for curve *2*. The circles shown along the solid curves are measured dielectric constant (the amplitudes are normalized) of alamethicin in 15% (curve *1*) and 23% (curve *2*) ethanol-dioxane. As shown, the agreement between experimental data and theoretical curves is reasonably good for both cases. The angle between the dipole moment and the major molecular axis is larger than we expected. The dipole moments of hydrogen bonded CO groups in α- or β-helices are only slightly tilted in relation to the major helix axis (Wada, 1962; Urry et al., 1975). In the case of alamethicin, the dipole axis deviates from the helix axis, indicating that the conformation of alamethicin is a complex one, including a certain amount of bending and even folding of the chain (Boheim, 1974; Mueller, 1980).

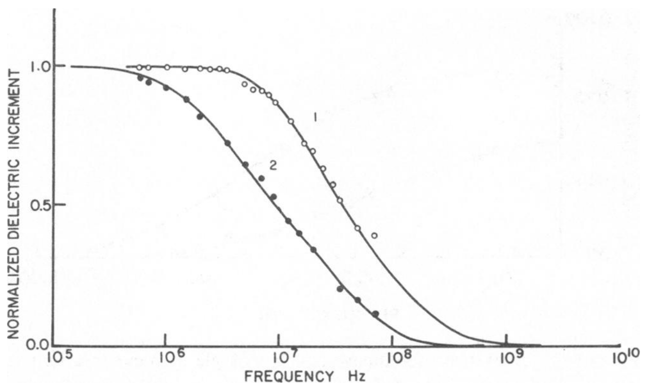

FIGURE 7 Normalized dielectric increments of alamethicin in different mixed solvents. The open circles (○) show the dispersion in 23% ethanol, and the curve was calculated with the assumption that the axial ratio of alamethicin is 9 (dipole angle 30°). The filled circles (●) were obtained with 15% ethanol, and the curve was calculated with $a/b = 10$ and the dipole angle 45°.

As discussed above, the axial ratios of alamethicin in various mixed solvents were found to be ~9–10. These values are used in the Perrin equation (Eq. 9) to calculate the length of the major axis of alamethicin. Fig. 6*b* shows some of the results of these calculations. This figure shows that the length of alamethicin varies from 37–38 to 20 Å as ethanol concentrations increase in mixed solvents. Clearly, shortening of alamethicin is associated with the increase in the magnitude of the dipole moment as seen in Fig. 6*a* and *b*.

DISCUSSION

As has been discussed, alamethicin has a dipole moment of 40–75 DU, depending on the polarity of the solvents. This value is considerably larger than the one predicted by Gordon and Haydon (1975). In view of the large dipole moment, it is reasonable to assume that the dipole moment may play an important role in voltage-dependent conductance, as discussed by several investigators previously (Baumann and Mueller, 1974; Hladky et al., 1974). The voltage-dependent conductance of a lipid bilayer doped with alamethicin can be defined by either one of the following equations (Gordon and Haydon, 1975)

$$G = G_0 \exp(zev/kT) \tag{11}$$

or

$$G = G_0 \exp(n\mu E/kt) \tag{12}$$

where e is the elementary charge, v is membrane potential, and n is an empirical parameter defining the slope of the conductance-voltage plots. Likewise, μ is the dipole moment and E is the electric field in statvolts (esu cgs). Rearranging Eq. 12, we obtain $kT\,[\ln (G/G_0)] = n\mu E$. Thus, the plot of $kT \ln (G/G_0)$ vs. E would be a straight line and its slope would be equal to the dipole moment. The conductance G is obtained from voltage-clamp experiments (Mueller and Rudin, 1968*a*), and ln G is plotted against the field E. To calculate the field strength E from the value of membrane potential, we used a length of 30 Å as the thickness of the hydrophobic layer of the membrane. Fig. 8 shows one of these plots. The linear portion is interpolated to zero field and the value of G_0 is obtained. Using the slope of this plot, we can calculate the value of dipole moment $n\mu$. The value obtained from Fig. 8 is ~680 DU. As discussed earlier, the dipole moment of alamethicin in lipophilic solvents is ~70 DU. Comparing these two sets of data, we found the value of n in Eq. 12 to be ~9–10.

Several points must be emphasized with regard to these results. (*a*) The above calculation is based on the assumption that the voltage dependence of membrane conductance is solely due to dipole moments. The contribution of discrete charges as monopoles is completely neglected. (*b*) The slope of the plots shown in Fig. 8 depends considerably on the concentration of electrolytes in the bathing solution (Roy, 1975). (*c*) The calculation of field strength E is subject to certain error due to the uncertainty of the membrane thickness. In any case, the value of the dipole moment obtained using Eq. 12 is always larger than the value of direct dipole moment measurements, indicating that the voltage-dependent conductance is due to aggregates of alamethicin molecules in lipid bilayers as suggested by Baumann and Mueller (1974). Also, it is most likely that the dipole moments in the aggregate are mostly parallel to each other.

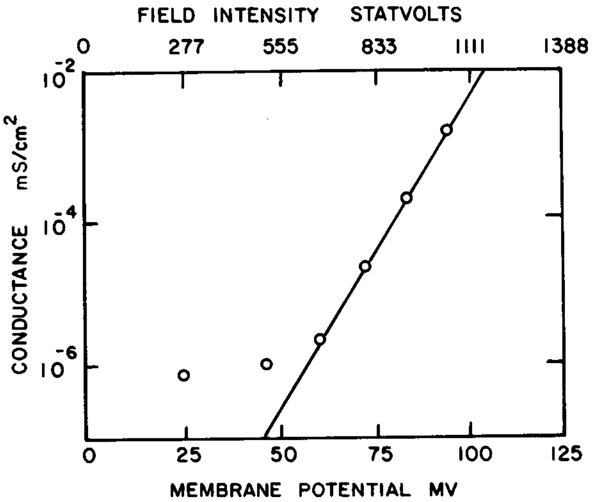

FIGURE 8 Voltage-dependent conductance induced by alamethicin in sphingomyelin bilayer membranes. The concentration of alamethicin is 10^{-6} g/ml. The upper scale (field strength E) is calculated on the assumption that the thickness of the hydrophobic layer of the membrane is 30 Å. The data were taken from Mueller and Rudin (1968). Other data by Roy (1975) and by Cherry et al. (1969) were also consulted.

The dimension of alamethicin determined using dielectric dispersion data is consistent with the results of other studies. The value of the effective length of alamethicin of 20 Å in a lipid medium agrees well with that obtained from monolayer studies, 16–20 Å (Chapman et al., 1969) and another value of 20.5 Å by Gordon and Haydon (1975). The decrease in the effective length of alamethicin with increasing polarity of solvents is in accord with the circular dichroism data of Jung et al. (1975). Their data indicate an increase in the alpha-helix content of alamethicin as solvent lipophilicity increases. This observation means that a decrease in the overall length and an increase in the dipole moment occurs, and that our data are in good agreement with their results.

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