Voltage-Induced Formation of Alamethicin Pores in Lecithin Bilayer Vesicles†

Arthur L. Y. Lau‡ and Sunney I. Chan*

ABSTRACT: The interaction of alamethicin with lecithin bilayer vesicles in the presence of a transmembrane potential difference has been studied by proton magnetic resonance spectroscopy. Asymmetrical vesicles with alamethicin trapped within the intravesicular compartment were prepared and the formation of ion channels were then triggered by imposing a suitable potassium ion gradient across the bilayer membrane in the presence of valinomycin. These alamethicin channels were found to permit the outward passage of europium ions into the extravesicular medium when the transmembrane potential is more positive for the inner compartment.

Alamethicin, an extracellular macrocyclic polypeptide from the fungus *Trichoderma viride* (Meyer and Reusser, 1967; Payne et al., 1970), has been shown to be capable of interacting with black lipid films to form voltage-gateable ion-conducting channels (Mueller and Rudin, 1968; Gordon and Haydon, 1972; Eisenberg et al., 1973). The solution properties of this antibiotic and its interaction with phospholipid bilayers have since been under extensive investigation by various methods (Chapman et al., 1969; McMullen et al., 1971; McMullen and Stirrup, 1971; Chelack and Petkau, 1973; Jung et al., 1975) in an effort to elucidate the mode of action of this oligopeptide in a bilayer system.

Application of nuclear magnetic resonance (NMR) spectroscopy to the study of alamethicin interaction with phospholipid bilayers was first performed by Hauser et al. (1970). These workers reported a broadening of the lipid ^{1}H NMR signals as well as a reduction in signal intensities caused by alamethicin. A later study by the present authors indicated that the oligopeptide actually interacts primarily with the bilayer in the region of the polar choline headgroups (Lau and Chan, 1974). The observed intensity loss was attributed to alamethicin-mediated fusion of vesicles in addition to specific interaction between the antibiotic molecules and these polar head groups. In a more recent study, we investigated the details of the fusion process (Lau and Chan, 1975). In that study we obtained evidence that some alamethicin molecules become translocated from the extravesicular aqueous medium into the intravesicular space during this vesicle fusion process. In addition, alamethicin was shown not to be incorporated into the hydrophobic core of the bilayer to any appreciable extent under the conditions of these experiments.

The study of the interaction of alamethicin with phospholipid bilayers would be incomplete, in our opinion, without an investigation into the action of alamethicin on the phospholipid bilayers when a potential difference is imposed across the bilayer. Since previous electrical studies on alamethicin properties in black lipid films have established that the ion-conducting channels are only formed in the presence of a transmembrane potential (Gordon and Haydon, 1972; Eisenberg et al., 1973), simulation of this situation in bilayer vesicle systems would appear necessary in order to correlate information obtained by magnetic resonance methods with those by electrical measurements. In this communication we therefore examine the effect of a transmembrane potential difference on the action of alamethicin on lipid bilayers.

Experimental Section

Materials. Alamethicin was obtained as a gift from Dr. G. B. Whitfield, Jr., of the Upjohn Co. (Sample No. U-22324. 8831-CEM-93.1) and was used without further purification.

L-α-Dipalmitoyllecithin (DPL) was purchased from Grand Island Biochemicals and was purified according to a published procedure (Robles and Van den Berg, 1969) before use. Chloroform and methanol, both used in column chromatography for the purification of DPL, were Matheson Coleman and Bell spectrograde products. Europium and lanthanum nitrates were purchased from Research Inorganic/Organic Chemical Corp. Potassium nitrate was a J. T. Baker reagent grade product. Deuterium oxide (99.8% ^{2}H) was from Stohler Isotope Chemicals, and A-grade valinomycin from Calbiochem. All chemicals were used as received.

Sample Preparation. Sonicated DPL bilayer vesicles of average diameter 300 Å were prepared using a Branson sonifier equipped with a microtip. A known quantity of purified DPL was weighed into a centrifuge tube, to which D_2O containing 2 mM potassium phosphate, 7 mM europium nitrate at pD 7.4 was added to give a suspension of about 30 mg of lecithin/ml of D_2O. Sonication with high power at level 4 for about 15 min gave a nearly transparent, colorless solution. This was centrifuged for 40 min at 12 000 rpm in a superspeed RC-2 centrifuge to remove metallic particles and residual multilayers from the small vesicles suspended in the supernatant. The latter was used in all subsequent experiments.

Asymmetrical DPL bilayer vesicles containing alamethicin trapped in the intravesicular medium were prepared by the following procedure. Measured quantities of alamethicin were weighed into a 1-ml volumetric flask and dissolved in an appropriate amount of D_2O containing 2 mM potassium phosphate, 7 mM europium(III) nitrate at pD 7.4. Known quantities of alamethicin and DPL vesicle solutions were then mixed and incubated at 70 °C for about 2 h. The incubated mixture

† From the Arthur Amos Noyes Laboratory of Chemical Physics, California Institute of Technology, Pasadena, California 91125. *Received September 30, 1975.* Contribution No. 5185. This work was supported by United States Public Health Service Grants GM-14523 and GM-22432 from the National Institute of General Medical Sciences.

‡ Danforth Foundation Predoctoral Fellow, 1971–1975.

[1] Abbreviations used are: DPL, dipalmitoyllecithin; ^{1}H NMR, proton magnetic resonance; EM, electron microscopy; DPPH 2,2-diphenyl-1-picrylhydrazl.

was dialyzed successively against three 100-ml solutions of 7 mM lanthanum(III) nitrate buffered at pD 7.4 with 2 mM potassium phosphate, for a total of not less than 3 h. NMR and EM experiments were performed with the dialysate. An ionic gradient was then created across the bilayer by adding to the external solution a sufficient amount of a 1 M potassium nitrate solution containing 2×10^{-4} M valinomycin at pD 7.4 so that the final solution in the extravesicular medium was 0.25 M in potassium nitrate. NMR and EM experiments were performed on this solution immediately afterwards.

^1H NMR Spectra. The Fourier transform (FT) spectra of sonicated lecithin bilayer vesicles were obtained with a Varian HR-220 NMR spectrometer operating at 220 MHz for protons and equipped with Fourier transform accessories interfaced to a Varian 620i computer. Intensities of signals were calibrated against a standard chloroform capillary treated with the free-radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Chemical shifts were measured against an external tetramethylsilane (Me_4Si) capillary without correction for variations in the bulk magnetic susceptibility. The ^1H NMR spectra of sonicated lecithin (in the absence of a shift reagent such as Eu^{3+}) consist of three peaks with chemical shifts of 3.21, 1.27, and 0.85 ppm downfield from the reference Me_4Si. Following earlier work, these peaks are assigned to the choline methyl ($N^+(CH_3)_3$), hydrocarbon chain methylene ($(CH_2)_n$) and aliphatic methyl (CH_3) protons, respectively. Sample temperature was maintained with a Varian 4540 temperature control unit, and was determined from the spectrum of a standard ethylene glycol sample.

Electron Microscopy. Electron microscopy was used to ascertain the size distribution of vesicles before and after the establishment of the ionic gradient. Specimens were prepared according to a procedure described in a previous paper (Lau and Chan, 1974) and were observed on a Philips 201 electron microscope operating at 60 kV.

Results and Discussion

Preparation of Asymmetrical Bilayer Vesicles and Creation of a Potential Difference Across the Bilayer. Sonicated bilayer vesicles are well suited for studies on membrane transport phenomena because they have distinct inner and outer media. In addition, a potential difference can be created across the bilayer membrane by setting up an ionic gradient in the presence of an ion-specific carrier.

In view of the strong tendency of externally added alamethicin to induce vesicle–vesicle fusion and the fact that ion translocation accompanies this fusion process, any ion transport via alamethicin pores formed concomitantly is completely masked by the predominant phenomenon of vesicle fusion. This difficulty can be circumvented if the alamethicin can be introduced into the intravesicular space of the bilayer vesicle. This can be conveniently accomplished, as we showed previously, by alamethicin-induced fusion, as the alamethicin becomes translocated into the intravesicular space during this process. Asymmetrical vesicles can then be prepared by purging the alamethicin molecules remaining in the extravesicular medium by dialysis after the fusion has subsided. Alamethicin molecules or aggregates thus trapped have been shown not to leak through the bilayer membrane and thus cannot induce further vesicle fusion.

When a potassium ion gradient is applied across the bilayer, a potential difference $\Delta\psi$ is created, which may be calculated from the Nernst equation:

$$\Delta\psi = \psi_{in} - \psi_{out} = -\frac{RT}{nF} \ln \frac{[K^+]_{in}}{[K^+]_{out}} \quad (1)$$

where $[K^+]_{in}$ and $[K^+]_{out}$ are concentrations of potassium ions in the inside and outside solutions of the bilayer, R is the universal gas constant, T the temperature, F the Faraday constant (=96487 C mol^{-1}), and $n = 1$ for the charge on a potassium ion. It is well known that an ion gradient results in a potential difference across the membrane barrier only if the membrane is permeable to the cation or the anion, but not to both. In our present experiment, valinomycin was added to the medium so that the membrane is permeable to potassium ions only. Valinomycin is an ion carrier capable of transporting monovalent cations, particularly potassium ions, across the bilayer. At electrochemical equilibrium, an electrical potential difference exists across the membrane, as given by eq 1. We have kept the outer solution more concentrated in potassium ions than the inner solution, so that the electrical potential inside is more positive. The sign of the electrical potential is an important factor in our experiments, since alamethicin was found to form ion-conducting channels in black lipid films only if the antibiotic is located on the positive side of the membrane.

In our experiments the concentration of potassium ions in the outer solution was 0.25 M. Since there were about 6 mM potassium ions in the inner solution (introduced as potassium phosphate buffer), the potential difference across the bilayer is calculated to be about 100 mV. The actual voltage may have been somewhat lower, as the ionic gradient causes the collapse of some small vesicles (vide infra) and results in some dilution of the outside salt concentration. However, in view of the rather small percentage of vesicles collapsed and the small intravesicular to extravesicular volume ratio, we believe this was not a serious problem in our experiments. This transmembrane potential should be sufficient to trigger alamethicin pore formation, since in analogous black lipid film experiments the gating potential is only about 50 mV at an alamethicin concentration of 10^{-6} M, and the apparent alamethicin concentration used in our experiments was on the order of 10^{-3} M.

We have used nitrate ion as the counterion in our experiments even though chloride is the most commonly employed anion in black lipid film work. Nitrate was selected because chloride is known to have a silent flux across lipid bilayers, which would complicate the interpretation of our experimental results.

Spectroscopic Indicator for Pore Formation. The formation of alamethicin pores in bilayer vesicles may be ascertained by the effect of a shift reagent such as europium(III) ions on the inner and outer choline methyl signals in the lipid ^1H NMR spectrum. The presence of europium(III) ions on one side of the bilayer membrane shifts the resonance of the choline methyl protons on that side upfield, without affecting the resonance due to choline headgroups in the other monolayer (Bystrov et al., 1971; Kostelnik and Castellano, 1972; Levine et al., 1973). Thus, if europium(III) ions are originally present in the intravesicular medium only prior to the creation of the ionic gradient, the spectral position of the inner choline signal could be used to indicate the leakage of europium ions and hence provide evidence for pore formation. Alamethicin pores, unlike many strongly selective ion-carriers, are rather cation nonspecific, although they do have somewhat varied conductances for different cations (Eisenberg et al., 1973). Previous experiments showed that the pores are even permeable to large ions such as tetraethylammonium, tetrabutylammonium, and chromium ions (J. E. Hall, private communication, and A. Lau, unpublished experiment). We, therefore, expect them to

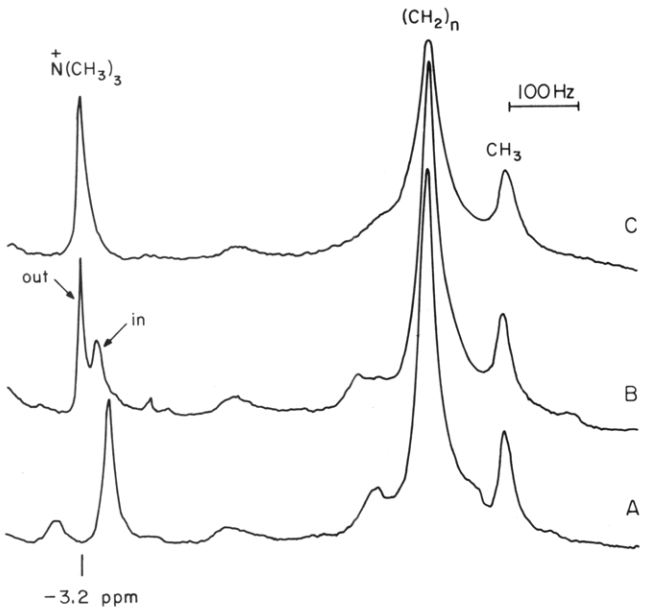

FIGURE 1: ^1H NMR spectra (220 MHz) of asymmetrical bilayer vesicles in D_2O at pD 7.4. (A) DPL vesicles mixed with 0.1% externally added alamethicin, fused for 2 h; 7 mM Eu^{3+} is present in both inner and outer media. (B) Same sample as in (A) but after dialysis against 7 mM $La(NO_3)_3$ solution. (C) Same sample as in (B) but after 0.25 M KNO_3 and 5×10^{-5} M valinomycin added to the external solution. All spectra taken at temperature of 70 °C.

be permeable to europium ions also.

In our experiments, we have put europium(III) ions initially on both sides of the bilayer vesicle by sonicating dipalmitoyllecithin in aqueous solution with europium nitrate. After alamethicin-induced fusion has subsided, the extravesicular europium(III) ions and any alamethicin left outside were replaced by an equimolar amount of lanthanum(III) ions by dialysis. Since lanthanum(III) ions have no shift effect on the lecithin ^1H NMR resonances, only the inner choline proton signal remains upfield. Also, because there is practically no europium ion in the outer solution, the free-energy difference for europium between the two media is almost infinite, i.e.,

$$\Delta G = RT \ln \frac{[Eu^{3+}]_{out}}{[Eu^{3+}]_{in}} - nF\Delta\psi \ll 0 \qquad (2)$$

and the extravesicular solution acts as an almost "infinite" sink for the europium ions. Thus, upon the formation of alamethicin pores, europium(III) ions would pass into the extravesicular solution, resulting in a downfield shift of the inner choline proton resonance.

Alamethicin Pores in Bilayer Vesicles. The effect of a 100 mV transmembrane potential on bilayer vesicles with entrapped alamethicin and europium ions is summarized in Figure 1. These data show that when a 0.25 M potassium ion concentration is present outside, europium ions do leak outwards, causing the inner choline ^1H NMR signal to shift downfield and to merge completely with the outer choline signal.

In this connection it should be mentioned that the time course of this downfield shift of the inner choline ^1H NMR signal is not observable by NMR spectroscopy, since at 7 mM rare earth ion concentration each vesicle of average diameter 400 Å contains only 600 or so europium ions, which would leak out in much less than a millisecond (judging from the magnitude of alamethicin pore conductance in black lipid membranes), once an alamethicin pore is open. This time scale is much too short for our method to follow.

Electron microscopy revealed that some of the smaller vesicles collapsed upon the addition of 0.25 M potassium nitrate to the extravesicular solution. Figure 2 shows the representative micrographs for sonicated vesicles before (A) and after (B) the alamethicin-induced fusion, and after the es-

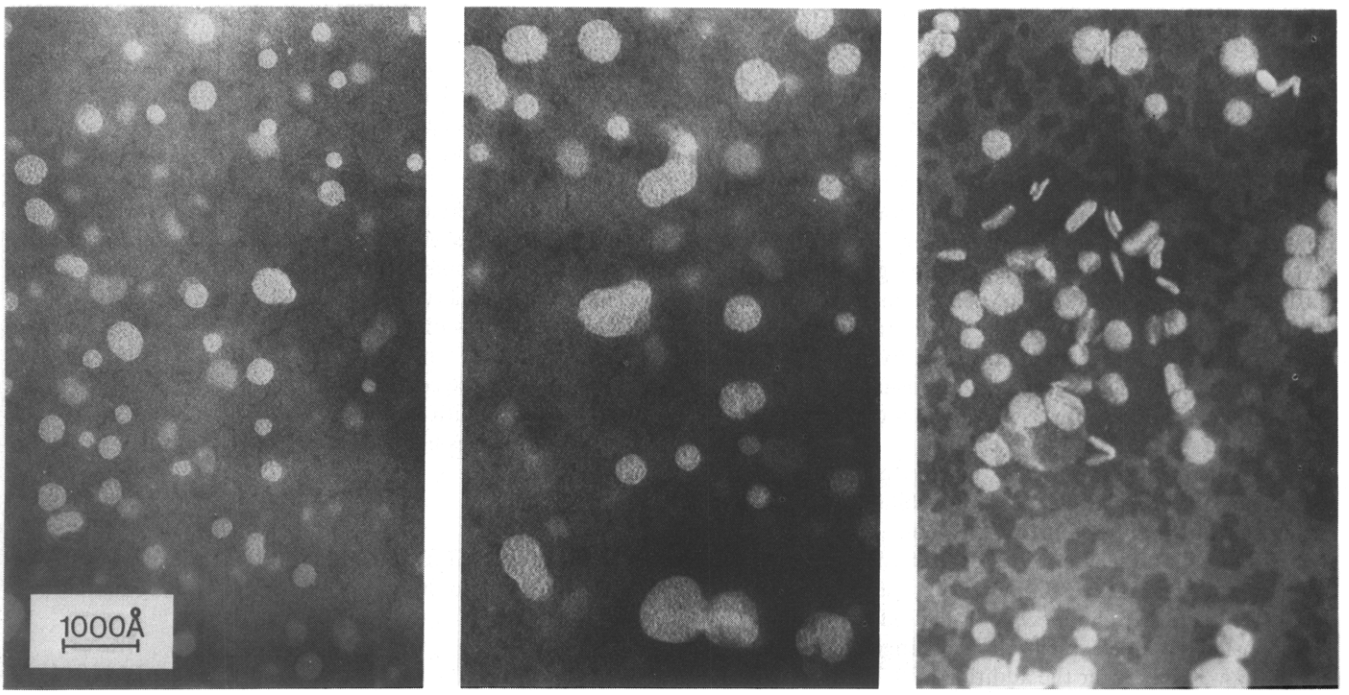

FIGURE 2: Representative micrographs for lecithin vesicle preparations. (A) Sonicated DPL vesicles in D_2O containing 7 mM $Eu(NO_3)_3$ in both inner and outer solutions. (B) DPL vesicles after fusion with 0.1% alamethicin and dialyzed against 7 mM $La(NO_3)_3$ for 3 h. (C) Same sample as in (B) but after 0.25 M KNO_3 added to the external solution.

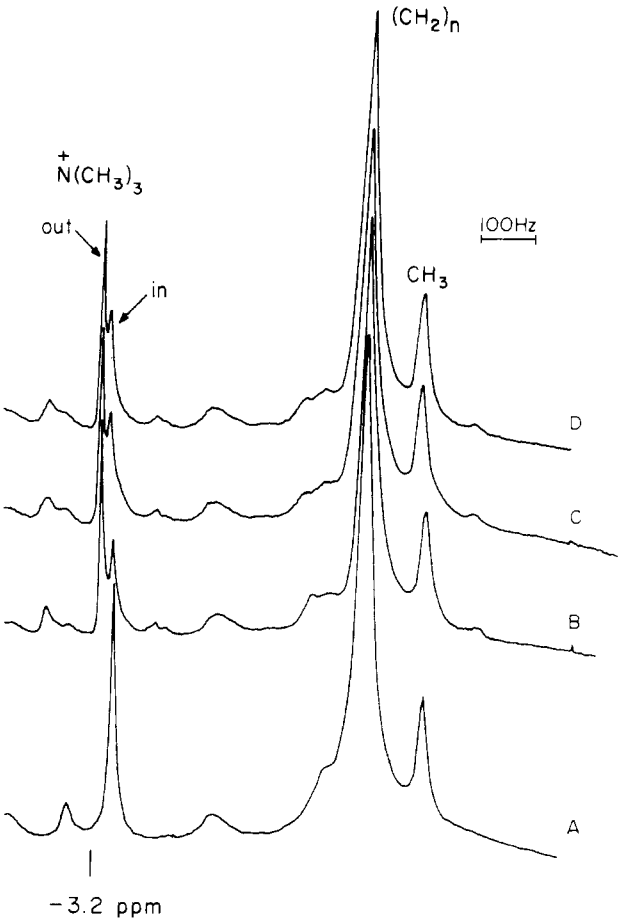

FIGURE 3: ^1H NMR spectra (220 MHz) of lecithin bilayer vesicles in D_2O at pD 7.4. (A) Vesicles prepared by sonicating DPL in a solution with 7 mM $Eu(NO_3)_3$. (B) Same sample as in (A) but after dialysis against 7 mM $La(NO_3)_3$ solution. (C) The dialyzed vesicle sample after 5×10^{-5} M valinomycin and 0.25 M KNO_3 were added to the external medium. (D) Same sample as in (C) but 30 min after the addition of KNO_3.

tablishment of an ionic gradient (C). The rice-shaped particles in Figure 2C represent collapsed vesicles. The fact that these collapsed particles are only observed in Figure 2C indicates that the vesicles are collapsed by the osmotic pressure created by the concentration gradient. Most of the larger vesicles of diameter 400–500 Å survived, however. This probably also explains why the fatty acid chain proton signal was somewhat broadened upon the addition of potassium nitrate.

The Role of Valinomycin. The importance of valinomycin as a charge coupler was demonstrated by repeating the experiment without valinomycin. No significant shift of the inner choline ^1H NMR signal was observed in the presence of the ion gradient.

Since valinomycin was used as a charge coupler in the above experiments, we had to rule out the possibility of europium ions being transported by valinomycin alone. As a control we therefore duplicated the previous experiment omitting the step of alamethicin addition. We found that the splitting between the inner and outer choline signals caused by europium ions in the inside remained essentially unchanged over a period of more than 30 min after the addition of 0.25 M of potassium nitrate to the extravesicular medium. These results are depicted in Figure 3. Some vesicle collapse was also observed with electron microscopy. Since no alamethicin was present, very little fusion occurred; hence, most of the uncollapsed vesicles were still sufficiently small (250–300 Å) to give a practically unaltered fatty acid proton signal line width after the addition of the salt. Thus, valinomycin does not transport europium ions across lipid bilayers. Being an ion carrier (Stark and Benz, 1971; Benz et al., 1973) rather than a pore-former, valinomycin is expected to be highly selective with respect to the size of ions it is capable of complexing. The cavity of valinomycin is too small to accommodate a europium ion even in its unhydrated state.

Conclusion

We have successfully created ion channels in alamethicin-containing bilayer vesicles by imposing an electrical potential across the bilayer membrane. Asymmetrical vesicles containing alamethicin and europium(III) ions in the intravesicular compartment were prepared by alamethicin-induced fusion followed by dialysis of the residual alamethicin and ions from the external medium. Subsequent application of a potassium ion gradient in the presence of an ion-coupler triggers the formation of ion-conducting channels with outward diffusion of europium(III) ions, provided that the polarity is of the correct sign and magnitude.

Apart from some broadening of the aliphatic methylene signal, which we have attributed to the collapse of some vesicles due to osmotic pressure created by the ion gradient, we have obtained no evidence for major perturbation of the bilayer, particularly disruption of the hydrocarbon interior upon the formation of ion channels. That is not to say, however, that some alamethicin aggregates have not in some way inserted into the hydrocarbon region of the bilayer, as the collapse of the splitting between the inner and outer choline methyl signal upon the onset of the pore-formation provides evidence for some transbilayer coupling, at least for the brief duration when the pores are open. That we have detected no effect is not totally surprising, perhaps, when we consider the total number of alamethicin molecules which might have been incorporated into the membrane under the condition of our experiments. If we assume that the partitioning of the oligopeptide is totally in favor of the bilayer, as opposed to the bulk solution, either on the bilayer aqueous interface or in the hydrocarbon interior, we estimate that no more than 30 or so alamethicin molecules are associated with each vesicle in our experiment. Since the number of lipid molecules per bilayer vesicle of diameter 400 Å is on the order of 2×10^4, we do not expect major effects on the observed methylene line width, as any perturbation on a few lipid molecules, even if large enough to be detected in some experiments, becomes averaged out over the large number of lipid molecules in a typical vesicle as a consequence of the long timescale of observation in an NMR experiment. The observed voltage-induced formation of the alamethicin ion channels should therefore be considered as an impurity effect. Only one or two channels with a lifetime of a fraction of 1 ms need to be formed in each vesicle to account for the results we have observed here.

Acknowledgment

We thank Dr. G. B. Whitfield, Jr., of the Upjohn Co., Kalamazoo, Mich., for his gift of a 25-mg sample of alamethicin.

References

Benz, R., Stark, G., Janko, K., and Läuger, P. (1973), *J. Membr. Biol. 14*, 339.

Bystrov, V. F., Dubrovina, N. I., Barsukov, L. I., and Bergelson, L. D. (1971), *Chem. Phys. Lipids 6*, 343.

Chapman, D., Cherry, R. J., Finer, E. G., Hauser, H., Philips, M. C., and Shipley, G. G. (1969), *Nature (London) 224*,

692.
Chelack, W. S., and Petkau, A. (1973), *J. Lipid Res., 14,* 255.
Eisenberg, M., Hall, J. E., and Mead, C. A. (1973), *J. Membr. Biol. 14,* 143.
Gordon, L. G. M., and Haydon, D. A. (1972), *Biochim. Biophys. Acta 255,* 1014.
Hauser, H., Finer, E. G., and Chapman, D. (1970), *J. Mol. Biol. 53,* 419.
Jung, G., Dubischar, N., and Leibfritz, D. (1975), *Eur. J. Biochem. 54,* 395.
Kostelnik, R. J., and Castellano, S. M. (1972), *J. Magn. Reson. 7,* 219.
Lau, A. L. Y., and Chan, S. I. (1974), *Biochemistry 13,* 4942.
Lau, A. L. Y., and Chan, S. I. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2170.
Levine, Y. K., Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C., and Robinson, J. D. (1973), *Biochim. Biophys. Acta 291,* 592.
McMullen, A. I., Marlborough, D. I., and Bayley, P. M. (1971), *FEBS Lett. 16,* 278.
McMullen, A. I., and Stirrup, J. A. (1971), *Biochim. Biophys. Acta 241,* 807.
Meyer, C. E., and Reusser, F. (1967), *Experientia 23,* 85.
Mueller, P., and Rudin, D. O. (1968), *Nature (London) 217,* 713.
Payne, J. W., Jakes, R., and Hartley, B. S. (1970), *Biochem. J. 117,* 757.
Robles, E. C., and Van den Berg, D. (1969), *Biochim. Biophys. Acta 187,* 520.
Stark, G., and Benz, R. (1971), *J. Membr. Biol. 5,* 133.

A β-Coupled Gauche Kink Description of the Lipid Bilayer Phase Transition†

Meyer B. Jackson

ABSTRACT: A statistical-mechanical model for a lipid bilayer was built around the assumption that β-coupled gauche kinks are the only nonstraight hydrocarbon chain configurations allowed. Packing of these chains was considered and expressions for the energy and entropy are developed based on those considerations. A solution was obtained which was not exact but was still accurate enough to be useful. In addition, an intermolecular potential was formulated which includes the attractive and repulsive forces between the hydrocarbon chains, the polar group repulsions, and hydrophobic forces. Combining the solution to the kinking model and the intermolecular potential, we obtain the total free energy of the lipid bilayer, which exhibits a first-order phase transition at a characteristic temperature. The theoretical heat of transition indicates that kinks alone can provide enough disorder for the transition to occur. Other physical quantities can be calculated, including order parameters. The order parameters calculated here give a picture of chains which are rigid near the polar group and more fluid near the methyl group.

As a model for biological membranes, lipid bilayers have been studied extensively. Though biological membranes are often more than half protein, their lipid portions have structure similar to that of pure lipids in lipid bilayers (Wilkins et al., 1971). The gel to liquid-crystal transitions seen in isolated membranes are also seen in isolated lipids (Melchior et al., 1970), and purified lipids show a transition of approximately the same magnitude, only much sharper (Hinz and Sturtevant, 1972a). In order to learn what can influence this transition and to gain a conceptual understanding of its nature, there has been some theoretical study. Assumptions concerning the transition can be tested by deriving theoretical relations which depend on those assumptions and comparing them with experimental results.

A variety of physical techniques have characterized the transition as an order–disorder transition with major changes occurring in the hydrocarbon chains. X-ray diffraction shows that the chains are straight, parallel, and arranged in hexagonal lattice in the gel phase. Above the transition temperature, the hydrocarbon chains have lost this order (Wilkins et al., 1971). Electron spin resonance (Hubbell and McConnell, 1971) and nuclear magnetic resonance (Urbina and Waugh, 1974) also show that a substantial loss of hydrocarbon chain order occurs during the phase transition.

Some investigators have suggested that, above the transition temperature, many or most of the hydrocarbon chains have β-coupled gauche kinks in them (Seelig and Niederberger, 1974; Seelig and Seelig, 1974; Trauble and Haynes, 1971). Here a kink is two gauche C–C bonds separated by one trans bond in an otherwise straight chain (Figure 1). There has been no direct detection of these kinks in bilayers, so that one can only speculate as to their importance. In this study, a theory was developed on the assumption that all nonstraight chain configurations and all the disorder of the liquid crystal can be accounted for by kinks of this type. A model is constructed and its approximate solution is used as the hydrocarbon chain disorder contribution to the free energy of the membrane.

Other theoretical work on this subject includes studies by Nagle (1973), Marcelja (1974), Jacobs et al. (1975), and McCammon and Deutch (1975). Nagle has used lattice statistics to exactly solve a planar array of infinitely long chains. In this study, a detailed picture of the critical point may have

† From the Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510. *Received December 15, 1975.* Supported by United States Public Health Service Grant GM-00711-18.