AN INVESTIGATION ON THE INTERACTIONS OF ALAMETHICIN WITH LECITHIN BILAYERS

Thesis by

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To my mother, brothers and sisters,

and in memory of my late father.

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ABSTRACT

The interaction of alamethicin, an extracellular macrocyclic polypeptide found in the fungus Trichoderma viride, with lipid bilayer systems in both unsonicated multilayer and sonicated bilayer vesicle states was investigated with nuclear magnetic resonance spectroscopy and electron microscopy techniques. We found that alamethicin is a surface active agent, which interacts primarily with the polar choline head groups of the lecithin. In its interaction with small sonicated bilayer vesicles ($\sim$300Å in diameter), alamethicin greatly facilitates the aggregation and subsequent fusion of these vesicles. Using europium ion, a lanthanide shift reagent, to probe the detail of this fusion process we found that during the alamethicin-mediated fusion some of the antibiotic molecules became translocated from the extravesicular aqueous medium into the intravesicular space. Alamethicin entrapped in this manner affects the choline methyl proton signals only from the inner half of the bilayer. No evidence was obtained for transmembrane coupling of the two halves of the bilayer or for effects of the incorporation of alamethicin molecules into the hydrophobic core of the bilayer in the absence of a transmembrane potential. In the presence of a potential difference across the bilayer, however, alamethicin is capable of forming ion-conducting channels. Such channels were found to be unidirectional for europium ions at least. A model for the voltage-induced formation of alamethicin pores in lipid bilayers is proposed and this model is discussed in the light of previously reported results of electrical studies on alamethicin-modified black lipid membranes.

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Chapter I

INTRODUCTION

The functioning of natural excitable membranes has long been thought to be due to some kind of voltage-dependent permeability changes in the membrane for certain ions (Hodgkin and Huxley, 1952). The propagation of the action potential in the nerve, for example, depends on the successive "turning-on and -off" of sodium and potassium ion conductances in the nerve membrane. The molecular mechanism of this voltage-induced change in permeability is not known, however. Indirect evidence has suggested that the increase in ion-conductance results from the opening of certain pores in the membrane (Hille, 1970). In recent years several small molecules such as alamethicin (Meyer and Reusser, 1967; Reusser, 1967), excitability-inducing material or EIM (Mueller and Rudin, 1963; Bean et al., 1969; Bean, 1972; Ehrenstein et al., 1970), gramicidin A (Sarges and Witkop, 1964; Hladky and Haydon, 1970,1972), and monazomycin (Mueller and Rudin, 1967; Muller and Finkelstein, 1972; Mauro et al., 1972) are known to induce, in black lipid membranes, voltage-dependent excitability not unlike that observed in certain biological membranes.

This thesis concerns an investigation of the interaction of alamethicin with lipid bilayer membranes. Alamethicin was chosen for this study because, unlike EIM, it is well-characterized. Its primary structure is known (Payne et al., 1970). Since this small molecule mimics many properties that are usually presumed for membrane proteins, its mechanism of action in lipid bilayers may serve as a useful model for

voltage-induced ion-transport in biological membrane systems. It is with this in mind that we undertook the studies described in this thesis.

1.1 Primary Structure of Alamethicin

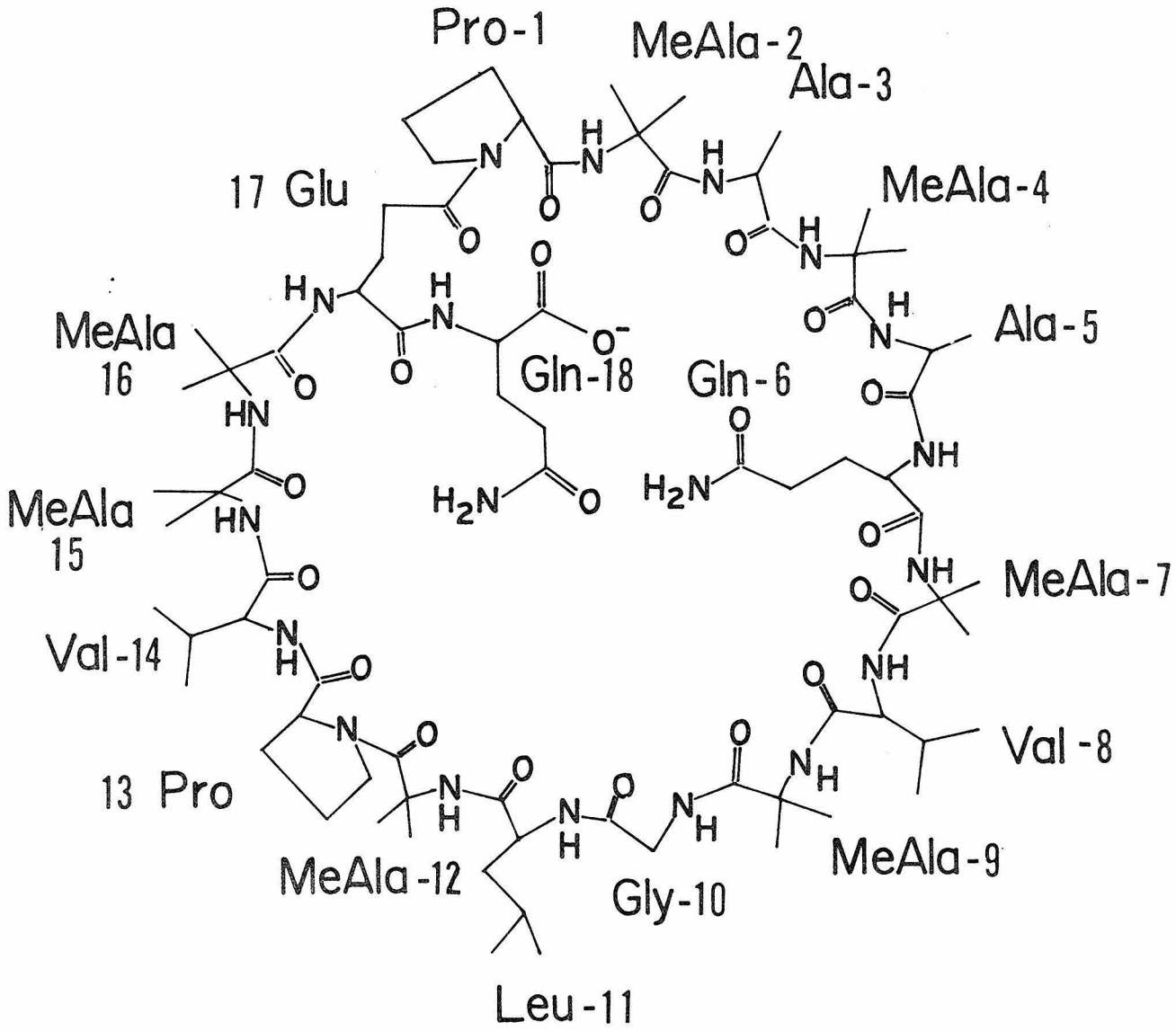
Alamethicin (antibiotic U-22324) is an extracellular peptide produced by the fungus Trichoderma viride. Its primary structure has been determined to be a macrocyclic polypeptide consisting of 18 amino-acid residues, including seven 2-methylalanines, two alanines, two prolines, two valines, two glutamines, a leucine, a glycine and a glutamic acid residue which carries a free carboxylic group. The molecular weight of the antibiotic according to this structure is 1771. The average diameter of the alamethicin ring is approximately 25Å. The ring is rather flexible, in contrast to many ion-carrier molecules which have relatively rigid conformations. When compressed alamethicin may assume an elongated conformation of about 36Å in length (Baumann and Mueller, 1974). The primary structure by Payne et al.* (Fig. 1) probably represents the structure of the major component of alamethicin which was later shown to be a mixture of three species separable by thin layer chromatography (Melling and McMullen, 1974). The structures of the two minor components have not yet been determined, but sedimentation analysis (McMullen and Stirrup, 1971) gives only one peak, indicating that the molecular weights of these three components must be reasonably close to one another. Probably

*Martin and Williams recently proposed another version of alamethicin's primary structure, see Chapter VII.

FIGURE 1

Primary structure of alamethicin (after Payne, Jakes and Hartley (1970), Biochem. J. 117, 757)

Structure of Alamethicin



the two minor components have similar, though not identical amino-acid content or sequence. Most of the research to date was performed with the unseparated mixture, and the so-called alamethicin pore in black lipid membranes is probably formed collectively by all three species rather than by just one of the three.

1.2 Electrical Properties of Alamethicin-Treated Membranes

Pressman (1968) showed that alamethicin is capable of inducing ion movement across certain biological membranes. Mueller and Rudin (1968) discovered that it can induce electrical excitability in lipid bilayers. The voltage-dependent gating phenomenon displays the characteristics observed in natural excitable cells. Gordon and Haydon (1972) showed that alamethicin aggregates form ion-conducting channels in black lipid films with discrete conductance states. Each conductance state has a typical lifetime of several milliseconds and typical conductance on the order of 10^{-10} mho. Both the lifetime and conductance depends on the type of lipid used to form the bilayer.

In a detailed study on the properties of the alamethicin pore, Eisenberg, Hall and Mead (1973) found, using bacterial phosphatidylethanolamine-decane membranes, that alamethicin aggregates are capable of forming ion-conducting pores in the membrane only if they are situated on the positive side of the bilayer. The so-called "characteristic voltage" (i.e., the voltage at which the membrane conductance reaches a certain pre-defined value, as defined by Eisenberg et al.) is dependent on the concentrations of both alamethicin and salt in the bulk solution, according to the following equations:

$$V_c = - V_a \ln \frac{c_a}{c_o} \qquad \text{(Eq. 1, Eisenberg } \underline{\text{et al.}}\text{, 1973)}$$

$$V_c = -V_{salt} \ln \frac{c_{salt}}{c_o'} \qquad \text{(Eq. 2, Eisenberg } \underline{\text{et al.}}\text{, 1973)}$$

where V_a and V_{salt} were experimentally determined by these workers to be 36 ± 3 mV and 16 ± 1.5 mV respectively. c_o and c_o' are two empirical constants. These four constants are actually products of several parameters such as membrane thickness, membrane conductance, temperature, etc.

At a voltage less than the characteristic voltage, V_c, the membrane conductance is indistinguishable from that of a bare membrane (bare membrane conductivity is $\sim 10^{-8}$ mho-cm^{-2}). Beyond V_c an abrupt rise in conductivity is observed. Typical voltage-current curves of an alamethicin-treated membrane is shown in Fig. 2. When the concentration of alamethicin in the bulk solution is very high, the characteristic voltage may become negative, and the current-voltage curve exhibits a region of negative resistance (Fig. 3).

The macroscopic conductance Λ of an alamethicin-modified membrane increases exponentially with applied voltage:

$$\Lambda = \Lambda_o \exp(V/V_o) \qquad \text{(Eq. 4, Eisenberg } \underline{\text{et al.}}\text{, 1973)}$$

where V_o is experimentally found to be 3.94 ± 0.05 mV, and is independent of the electrolyte used. The mechanism of pore-formation is voltage-gated, and the higher conductance of the membrane beyond the characteristic voltage merely reflects an increase in the number of open

FIGURE 2

Family of current-voltage curves in symmetric 0.1 M NaCl solution with different alamethicin concentrations: (a) 5×10^{-8} g/ml, (b) 10^{-7} g/ml, (c) 2×10^{-7} g/ml, (d) 5×10^{-7} g/ml, (e) 10^{-6} g/ml and (f) 1.5×10^{-6} g/ml. Rate of voltage-sweep: 12 mV/sec. The baseline current is the sum of the capacitive current and the current through the bare membrane. (Reproduced from Eisenberg et al., J. Membrane Biol., 14, 143 (1973) by permission of the authors)

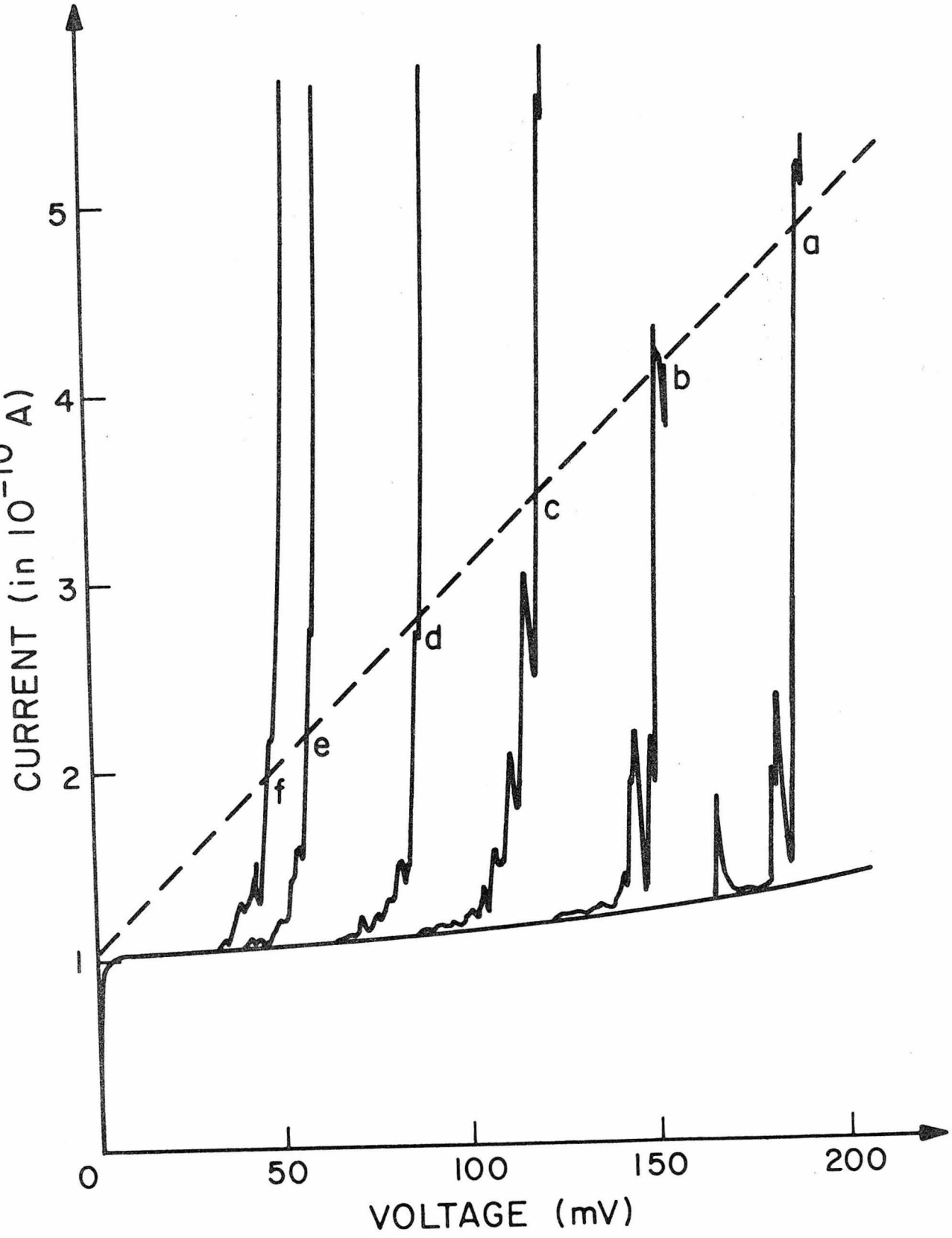
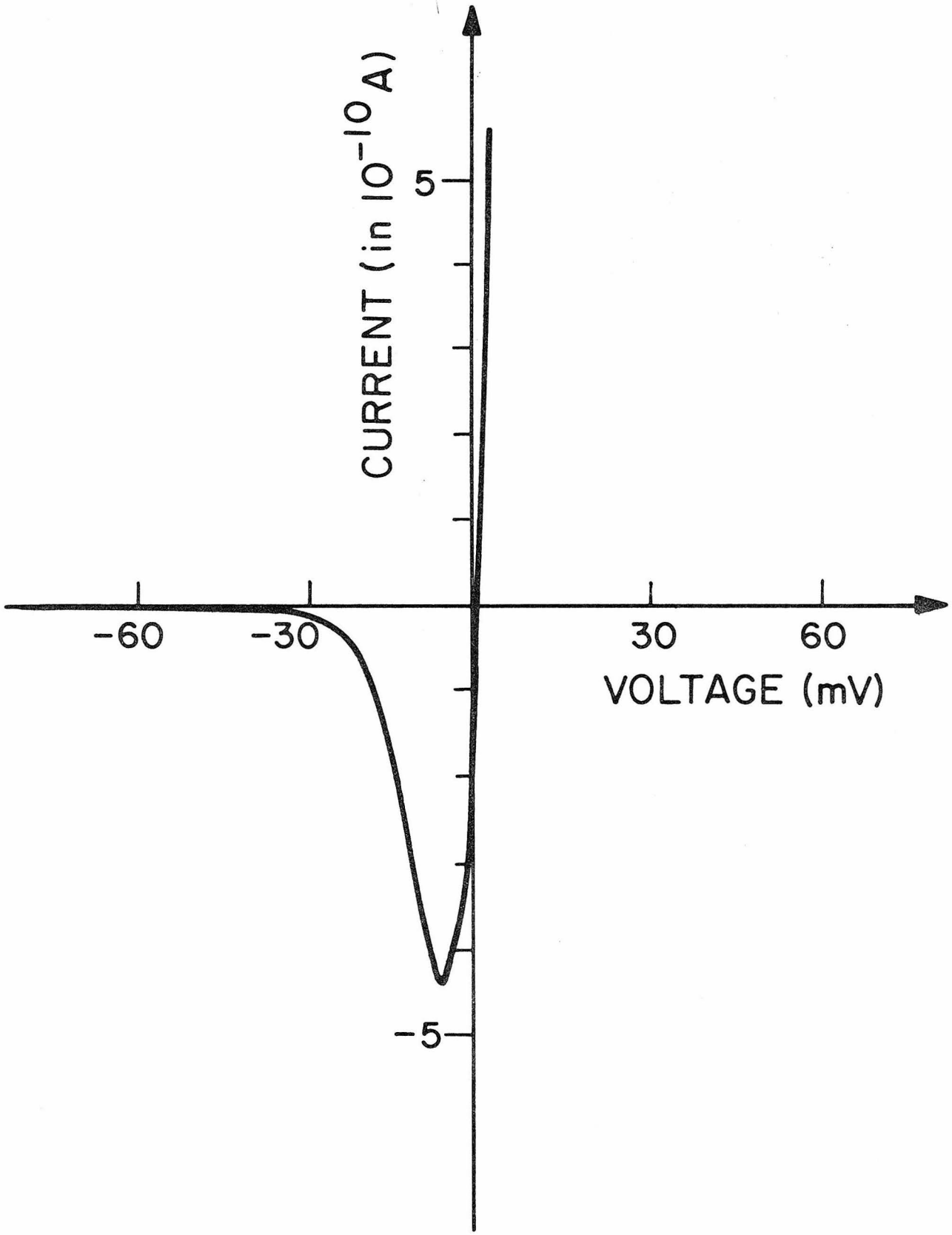

CURRENT (in 10^{-10} A)
5
4
3
2
1
0
50
100
150
200
VOLTAGE (mV)
a
b
c
d
e
f

FIGURE 3

Voltage-current curve of a PE-decane membrane, $0.5 \times 10^{-2} cm^2$ in area with 1.0 M NaCl on both sides and 10^{-5} g/ml alamethicin added to one side only. (Reproduced from Eisenberg et al., J. Membrane Biol. 14, 143 (1973) by permission of the authors)


CURRENT (in 10^{-10} A)
5
-5
-60
-30
30
60
VOLTAGE (mV)

pores at a given time. The conductance of each individual pore is ohmic; the current through a pore increases (approximately) linearly with voltage. Each alamethicin pore can have five most probable, discrete conductance levels with many intermediate and higher conductance levels that are simply sums of two or more of these five states. These five basic states are not simple multiples or sums of one another, however, implying that all five states are intrinsically associated with a single physical entity called a pore.

Alamethicin adsorbs reversibly to the membrane surface. Eisenberg et al. (1973) determined that the conductance at a fixed voltage depends on the ninth power of alamethicin concentration and on the fourth power of salt concentration in the aqueous phase. These values are in contrast to a sixth power dependency on both alamethicin and salt concentrations observed by Mueller and Rudin (1968). The discrepancy probably lies in the difference in the most probable pore-forming aggregate in their respective experiments. Eisenberg et al. used bacterial phosphatidylethanolamine (PE)-decane membrane at 23°C, whereas Mueller and Rudin used sphingomyelin-tocopherol membrane at 35°C. We believe this difference is enough to account for the discrepancy between the two sets of results.

Unlike most ion-carriers such as valinomycin and nonactin which are highly ion-selective, alamethicin pores are quite cation-nonspecific although they do show different conductance values for different cations and have a preference of cations over anions.

1.3 Conformational Studies on Alamethicin

McMullen, Marlborough and Bayley (1971) have examined the conformational properties of alalmethicin by optical rotary dispersion and circular dichroism in a variety of solvents. No effect of monovalent cations on the spectra was observed, but the spectra were shown to be strongly solvent dependent. For example, in aqueous solvent containing 10% ethanol the spectra indicated a less ordered structure than found in pure ethanol. It appears that the conformation of alamethicin becomes more ordered in more hydrophobic solvents. Jung, Dubischar and Leibfritz (1975) found, using CD and ^{13}C nmr techniques, that alamethicin has a highly stabilized non-polar α-helical part. Concentration dependence of the CD-spectrum of alamethicin revealed aggregation phenomena (we shall again describe this aspect in Chapter VII when we discuss the solution properties of the polypeptide). These workers claimed that four different conformations can be characterized by CD spectra depending on the solvent used. Such conformation changes in media of different dielectric constants may have some implications on the mechanism of pore-formation, which we shall take up in Chapter VIII.

Theoretical calculations on alamethicin conformation was performed by Burgess and Leach (1973) who determined that three of the seven 2-methylalanines occur in a six-residue α-helix and three others at the chain reversals. Johnson (1973) proposed that the minimal energy conformation of alamethicin consists of an α-helix from residues 1 through 9, a bend at 10 and 11, and a stretched out chain from 11 to

the end, rejoining residue 1 at residue 17. Neither of these conformations suggests any strong cation-antibiotic complex or cation-specificity. That such complexes and specificities are absent may be correlated to the nmr results presented in Chapter VII (vide infra). X-ray crystallographic determination of the secondary and higher order structures of alamethicin has not yet been successful.

1.4 Partition of Alamethicin between Aqueous and Lipid Phases

Chelack and Petkau (1971) reported a partition ratio of 17:1 for alamethicin between lipid and aqueous media. However, in view of the strong tendency of the oligopeptide to promote vesicle fusion (see Chapters IV and V), we believe that their result can be better interpreted as the ratio of alamethicin entrapped inside the vesicles as a result of fusion to those left in the extravesicular medium. Assuming that their measurements were accurate, this would mean that, at equilibrium, about 95% of the alamethicin molecules become translocated into the intravesicular space. The dissipation of the high surface energy of the vesicles probably accounts for the free-energy required for such a massive translocation.

1.5 General Purpose of Thesis Work and Thesis Outline

A knowledge of the mode of interaction of alamethicin with phospholipid bilayer systems is essential for understanding the action of this antibiotic, and for correlating data obtained from black lipid film experiments with the properties of alamethicin in free solutions. Among the various techniques which might be employed in this study,

such as X-ray diffraction and differential scanning calorimetry (Chapman et al., 1969; Levine et al., 1973), electron spin resonance spectroscopy (Finer et al., 1969; Levine et al., 1973), circular dichroism and fluorescence techniques (Case et al., 1974), unique advantages are offered by nuclear magnetic resonance spectroscopy by virtue of its sensitivity to structural details and environmental changes. This sensitivity has been exploited in many nmr investigations of the structural and dynamic properties of lecithin bilayers (see, for example, Penkett et al., 1968; Chan et al., 1971; Lee et al., 1972,1973; Horwitz et al., 1972; Levine et al., 1972; Michaelson et al., 1973; Feigenson and Chan, 1974; Seiter and Chan, 1973; Seelig and Seelig, 1974a,b; Fujiwara et al, 1974; Gent and Prestegard, 1974). More recently, proton magnetic resonance (pmr) spectroscopy has also been used to monitor the interaction of various oligopeptides with lipid multilayers and sonicated bilayers (Hauser et al.,1970; Hsu and Chan, 1973; Chang and Chan, 1974; Yu et al., 1974; Kumar et al., 1975).

This thesis describes a pmr study of the interaction of alamethicin with dipalmitoyl lecithin bilayers in both the unsonicated multilamellar (Bangham and Horne, 1964) and sonicated bilayer vesicle states (Huang, 1969). That both bilayer systems must be investigated in order to correlate the results with the data from black lipid membrane experiments is evident from the fact that the unsonicated liposomes and sonicated vesicles offer two different, albeit interrelated, model membrane systems, each with some unique advantages in membrane studies. In terms of bilayer structure, these two model systems differ primarily in their surface curvatures (Sheetz and Chan, 1972). Unsonicated multilayers, with a small surface curvature, bear the greater resemblance to biological

cell membranes and artificial black lipid films. Vesicle curvature can have a profound influence on the molecular packing of the phospholipid molecules in a bilayer (Seiter and Chan, 1973; Chan et al., 1973; Lichtenberg et al., 1975). More specifically, it has been argued that the packing arrangements of these molecules in multilayers is more regular than in bilayer vesicles about 300Å in diameter. This difference in the molecular packing of the lipid molecules is reflected most dramatically in the segmental motions of the fatty acid chains. Whereas the segmental motion of the hydrocarbon chain is relatively restricted in the case of flat bilayers, their motion is much freer in small bilayer vesicles. The state of motion of the hydrocarbon region in sonicated vesicles is thought to very nearly resemble that of a hydrocarbon liquid. In view of this pronounced effect of vesicle curvature on bilayer structure, a comparative study of the interaction of alamethicin with both sonicated and unsonicated lecithin bilayers appeared necessary.

By virtue of their large surface curvature, small bilayer vesicles are expected to be intrinsically less stable thermodynamically than their unsonicated multilamellar counterpart. In fact, evidence is accumulating to indicate that small bilayer vesicles do tend to undergo vesicle-vesicle fusion (Prestegard and Fellmeth, 1974) although the rate of such fusion can be somewhat controlled by appropriate choices of experimental conditions such as temperature and ionic strength. In the presence of a surface-active agent such as alamethicin this process of vesicle fusion becomes more probable. My investigation of alamethicin-promoted vesicle fusion will be described in detail in Chapter V.

Despite the fact that sonicated bilayer vesicles are thermodynamically much less stable than unsonicated multilayers, they offer a unique advantage as a model membrane system in having well-defined inner and outer media--this property is completely lacking in multilayers. This makes sonicated vesicles especially suitable for studies of membrane-related ion-transport phenomena. In Chapter VI we shall discuss the possibility of pore formation by alamethicin in bilayer vesicles when a transmembrane potential difference is present.

The general solution properties of alamethicin will be discussed in Chapter VII. In that chapter we shall investigate the phenomenon of aggregation of alamethicin in solutions of different ionic strengths.

Chapter VIII is the culmination of all the preceding chapters. Based on the information obtained in earlier chapters, a molecular model of alamethicin pore formation is presented. The model will be analyzed in light of the data reported earlier on black lipid film experiments (Eisenberg et al., 1973).

Chapter II

MATERIALS AND METHODS

2.1 Techniques

2.1.1 Fourier Transform and Delayed Fourier Transform NMR Spectroscopy: A General Description. An important characteristic of conventional nmr spectrometers is that they scan the spectra by sweeping either the field or the frequency through the region of nmr absorption. With this mode of operation, only a very narrow band of frequencies contributes to the spectrum at a given time. The disadvantages of this method are obvious: the spectral quality is sweep-rate dependent, and has a low signal-to-noise ratio. For samples with low spin concentrations the spectra may be completely obscured by random noise, making their quantitative analysis impossible. This problem may be somewhat alleviated by the method of multichannel excitation and detection. The computer-averaged-transient method (the CAT method) uses multiple scanning of a given spectroscopic frequency range to improve the spectrum's signal-to-noise quality. The result of each scan is stored in a multichannel analyzer, and when the results of N scans are added, the spectrum improves by a factor of $N^{1/2}$. However, with very dilute samples, notably those used in natural abundance ^{13}C nmr experiments, an impossibly large number of scans would be required for a decent spectrum. The Fourier transform method, on the other hand, accomplishes essentially the same thing as the CAT method, only in a more satisfactory manner, and is used widely in modern nmr instrumentation.

In a Fourier transform experiment, a strong radiofrequency pulse excites the entire range of precession frequencies of the chosen nuclear species, and, after the radiofrequency pulse is turned off, each of these excited species begins to lose its magnetization. The rate of the free induction decay is determined by magnet inhomogeneity and by various relaxation mechanisms within the system; the decay signal is detected by the receiver. It has long been known (Lowe and Norberg, 1957) that under certain easily satisfiable conditions, the Fourier transform of this free induction decay is identical to the steady-state slow-passage absorption spectrum. The transform process thus plays the role of the multichannel analyzer in the CAT method, but saves the use of a large number of narrow band filters and detectors.

The advantage of Fourier transform method lies in the much shorter time needed to achieve a signal-to-noise ratio comparable to that observed with a CAT. Each sequence of radiofrequency pulse and free induction decay normally requires a time on the order of seconds whereas a slow-passage scan takes 100-1000 seconds. Since the improvement in sensitivity increases as the square root of the number of scans, multiple scanning takes at least 100 times longer to achieve a sensitivity comparable to that achieved by Fourier transform. Experiments which take days or weeks to do otherwise may be accomplished in only minutes or hours with the Fourier transform method. This advantage is even greater when samples which change with time are used.

The Fourier transform method offers more than just an improvement in experiment time. For example, with a normal pulse nmr spectrometer,

the free induction decay signal is the sum of the signals from all species present in the sample. With the Fourier transform, separate determination of relaxation rates of individual species becomes possible.

A variation of this Fourier transform technique permits the filtering of certain huge, broad resonances which may otherwise obscure the analysis of sharper signals from the transformed spectrum. This is accomplished by the delayed Fourier transform technique (Seiter et al., 1972). This technique is based on the fact that the broad resonances have much shorter spin-spin relaxation times (T_2) than narrower ones. After a radiofrequency pulse is applied and subsequently turned off, each component of the transient signal begins to decrease in magnitude; the rate of decrease is inversely related to its spin-spin relaxation time. After a certain time, τ , the magnetization of each component species will have decayed to $\exp(-\tau/T_2)$ of its initial value, according to the equation

$$M(t) = M_0 \exp(-t/T_2)$$

Thus, after a time $\tau = 5$ units, say, a signal with T_2 equal to 1 time unit would only have about 1% of its original value remaining, whereas a signal with a T_2 equal to 100 units still retains 95% of its initial magnetization. Thus if an acquisition delay time of 5 units is introduced between the end of the radiofrequency pulse and the start of data accumulation, much of the broader signal ($T_2 = 1$) is filtered out, while the sharper resonance ($T_2 = 100$) is only minimally affected.

This technique is especially useful in nmr studies of unsonicated multilayer systems. Typical nmr spectra of unsonicated lecithin multilayers have an exceedingly broad and intense (half-width $\sim$3000 Hz, intensity $\sim$80% of total protons present) signal due chiefly to the methylene chain protons, with two narrower (half-widths $\sim$100-300 Hz) signals from the choline and terminal methyl protons superimposed on the broad resonance. The delayed Fourier transform technique permits filtering out the broad methylene resonance while retaining the two methyl resonances for quantitative analysis. The advantage of the DFT method is exploited in Chapter III, where we study the interaction of alamethicin with unsonicated dipalmitoyllecithin multilayers.

2.1.2 NMR Instrumentation. Continuous wave pmr spectra, recorded at 220 MHz, were obtained on a Varian HR-220 spectrophotometer with a superconducting magnet equipped with a variable temperature accessory, operating in the field sweep mode.

Fourier transform (FT) nmr spectra were obtained either on a Varian HR-220 or a Varian XL-100 spectrometer, equipped with Fourier transform accessories and interfaced to a Varian 620i computer.

Pulse nmr measurements were made on a system consisting of a Model BA Pulse Programmer, Model BK Superhet Interface and Model BR 250 watt transmitter, all purchased from Tomlinson Research Instruments Corp., with an electromagnet operating at 54 MHz for protons (12.7 kilogauss). Probes for this system were of homemade construction by Dr. G. W. Feigenson. Detailed description of probe construction and basic circuitry can be referred to elsewhere (Feigenson, 1974). Spin-spin

relaxation times were recorded by photographing the oscilloscope traces of the phase-detected free induction decay.

In all experiments sample temperature was controlled with a Varian 4540 variable temperature controller and was determined from the spectrum of a standard ethylene glycol sample . Intensities of the pmr signals were calibrated against a standard chloroform capillary treated with the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), which was in turn calibrated against a standard solution of tetramethylammonium bromide. Chemical shifts were measured against an external tetramethylsilane (TMS) capillary without correction for changes in the bulk magnetic susceptibility.

For the sake of clarity, procedures for nmr sample preparation will be described separately in each appropriate chapter.

2.1.3 Negative Staining Electron Microscopy. Electron microscopy involves the production of images of adequate contrast with the background at a magnification sufficient to reveal the relevant details of the object under investigation.

Image formation involves diffraction, the scattering of light or electromagnetic radiation by the object, and the focusing of the scattered light into an image. Thus the object must modify the electron beam to a sufficiently different degree than the background does in order to be observable under an electron microscope. In many cases the interactions of the object and background with the electron beam are not sufficiently different to produce observable effects. In that event, either the object or the background must be systematically

modified to yield useful information about the object--a process called "staining".

Positive staining involves the combination of an electron-dense material with the object of interest so that the object appears dark in the electron micrograph against a light background. With positive staining one runs the risk of modifying the object of interest with the staining reagent. On the other hand, if the staining involves combining the electron-dense material with the background but not with the object, the opposite situation is observed. The negatively stained object appears transparent in the micrograph against a dark background (see Figs. 9 and 20 for illustration). Negative staining is advantageous in that the staining reagent interacts mainly with the background material, and the object under examination remains more or less unmodified; the image produced in this way corresponds more closely to the true picture.

In our experiments we have used the negative staining technique to see the vesicle sizes and state of aggregation. Parlodion film was employed as the supporting background material because it has an excellent wetting property. The sensitivity of the parlodion film to destruction by the electron beam was overcome by coating the film with an extremely thin layer of carbon ($\sim 10\text{Å}$). Since carbon is transparent to electron beams, no alteration of background diffraction is produced by its presence. Resolution of the specimen is only minimally affected since the layer of carbon is thin. Parlodion film prepared in this way is more susceptible to phosphotungstic acid (the staining reagent) than are the lecithin vesicles. When spread on a sheet of parlodion dotted

with vesicles, the phosphotungstic acid forms a continuous matrix of electron-opaque background, leaving the area occupied by the vesicles electron-transparent.

2.1.4 Preparation of Specimens for Electron Microscopy. Immediately before and after each nmr experiment, a portion of the vesicle solution was diluted to 1-5 mg in lecithin per ml D_2O. In cases where alamethicin concentration in the solution was high, further dilution was often necessary to disperse the vesicles so that they became more easily observed under the electron microscope. A drop of this diluted vesicle solution was then applied to a 200-mesh copper grid coated with parlodion sprayed with a thin film of carbon. After $\sim$30 seconds the excess liquid was blotted out, and the grid was washed successively by three drops of the staining solution, 2% phosphotungstic acid (PTA) at pH 7.4, with the grid tilted at an angle of approximately 60° to the horizontal. With the grid held horizontal, an additional drop of the PTA solution was then applied for 30 seconds. The excess liquid was blotted off by a piece of fine filter paper and the grid allowed to dry prior to observation under the electron microscope.

Grids prepared according to the above procedure were observed on a Philips 201 electron microscope operating at 60 kilovolts at mag levels 11 or 12 (actual magnification on screen 70000 or 100000, and on camera 20600 or 29600 respectively).

Vesicle size distributions were determined by sizing approximately 500 vesicles from the electron micrographs. Polystyrene beads of average diameter 1000 ± 50Å were used to calibrate the electron micrographs.

2.2 Materials

Alamethicin was obtained as a gift from Dr. G. B. Whitfield, Jr., of the Upjohn Company, Kalamazoo, Michigan (Samples No. U-22324.8831-CEM-93.1 and 93.3) and was used without further purification.

L-α-Dipalmitoyllecithin was purchased from General Biochemicals. It was purified with silicic acid column chromatography (Robles and Van den Berg, 1969). One hundred grams of silicic acid was used per gram of lecithin. A 2.5 × 50 cm column was used, and the lecithin was eluted with 400 ml of $CHCl_3$, followed by 400 ml of CH_3OH-$CHCl_3$ mixture in a 1:9 ratio, and finally with 1500 ml of CH_3OH-$CHCl_3$ (9:1). The lecithin came out in the last eluent. After rotary evaporation of the eluting solvent a white product was obtained, and thin layer chromatography of it gave a single spot.

Deuterium oxides (100% ^2H and 99.8% ^2H), used without further purification in the preparations of unsonicated multilayer and sonicated bilayer vesicles, respectively, were purchased from Stohler Isotope Chemicals. Chloroform and methanol were Matheson Coleman and Bell spectrograde products. Europium and lanthanum nitrates were from Research Organic/Inorganic Chemical Corp. Valinomycin, A-grade, from Calbiochem was also used without further purification. Phosphotungstic acid ($P_2O_5 \cdot 24\ WO_3 \cdot xH_2O$, used as an EM staining reagent) was from Mallinckrodt Chemical Works. Potassium nitrate and sodium chloride were Baker analyzed reagent grade products.

Chapter III

INTERACTION OF ALAMETHICIN WITH UNSONICATED LECITHIN MULTILAYERS

3.1 Introduction

We have monitored the effect of alamethicin on unsonicated lecithin bilayers using two nmr techniques: conventional pulse nmr and the recently developed delayed Fourier Transform (DFT) nmr method. The continuous wave pmr spectrum of unsonicated lecithin bilayers above the thermal phase transition consists of two relatively sharp (100-200 Hz halfwidths) signals arising from the choline methyl and terminal methyl protons superimposed on a significantly broader ($\sim$ 3000 Hz) resonance. Analysis of the sharper signals is obscured by this latter broad resonance which was recently shown to be a sum of three broad lines due to some of the choline and terminal methyl protons and all of the methylene protons in the fatty acid chains (Feigenson and Chan, 1974). This has hampered nmr studies with unsonicated lecithin bilayers in the past. This problem can be circumvented by the use of the delayed Fourier Transform method. In DFT nmr spectroscopy one exploits the significantly shorter transverse relaxation time of a very broad signal compared with those for sharper resonances. Thus if a suitably chosen receiver dead-time is introduced between the end of the applied radiofrequency pulse and the start of data collection, much of the broader resonance can be filtered out after Fourier Transform of the truncated free induction decay (See Chapter II for a general description of the DFT method). When applied to an unsonicated lecithin multilayer system, this method in essence isolates the narrower methyl signals and permits accurate

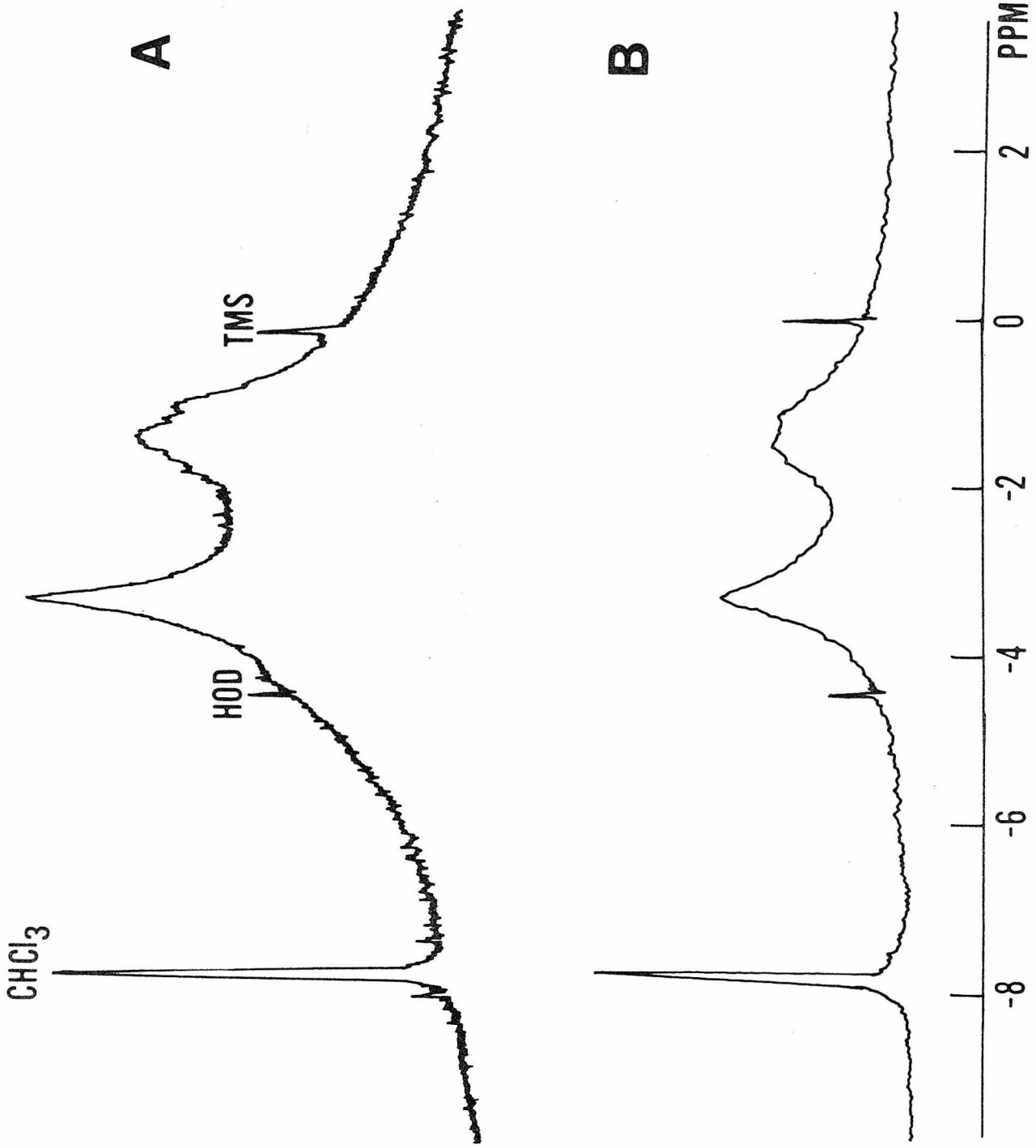
intensity and linewidth measurements on them (Fig. 4). Two nuclear spin probes, located in quite different regions of the bilayer, thus become available to explore the structural and dynamical properties of the surface and interior of the lecithin bilayer. To augment these measurements, the chain methylene protons, which account for about 80% of the total protons in the system, are readily monitored by conventional pulse nmr. The utility of these techniques in bilayer nmr work has been adequately documented (Chan et al., 1971,1973; Hsu and Chan, 1973).

3.2 Sample Preparation

A known amount of the purified lecithin was weighed into an nmr tube with an attached vacuum ground glass joint. About 0.1 - 0.2 ml of spectrograde chloroform was then added to dissolve the lecithin. For samples containing alamethicin, the polypeptide was added prior to the addition of chloroform. The sample was then mixed by vortexing and let stand overnight to equilibrate. After capillaries containing respectively $CHCl_3$ doped with the free-radical 2,2-diphenyl-1-picrylhydrazyl (intensity standard), silanor C (chemical shift reference) and ethylene glycol (temperature standard) were added to the sample, the lecithin-chloroform solution was vacuum-pumped for 48 hours to remove the solvent. Warming the sample to 50-60° during evacuation was found to be necessary to drive off the last traces of chloroform and methanol used in the chromotography purification. Deuterium oxide (100% ^2H) was then transferred into the sample to obtain an approximately 25% (w/v)

FIGURE 4

Typical pmr spectra (220 MHz) of unsonicated multilayers in D_2O. (A) Continuous wave spectrum of a dipalmitoyllecithin multilayer sample at 48°. (B) Delayed Fourier transformed spectrum of the same sample. Receiver dead time = 346 μseconds.


A
B
$CHCl_3$
HOD
TMS
-8
-6
-4
-2
0
2
PPM

lecithin suspension. After the tube was sealed under nitrogen at slightly less than atmosphere pressure, it was heated in a water bath to about 60°, and the suspension contained in it was homogenized by extensive agitation with a vortex mixer.

3.3 PMR Instrumentation

The delayed Fourier transform spectra of these multilayer samples were obtained with an HR-220 nmr spectrometer. A data collection delay time of 346 μsec was introduced in order to filter out the broad methylene component in the pmr spectrum. The intensities of the choline methyl and terminal methyl proton resonances were measured against an external chloroform-DPPH intensity standard, and were corrected for the loss due to receiver dead time introduced into the spectrometer.

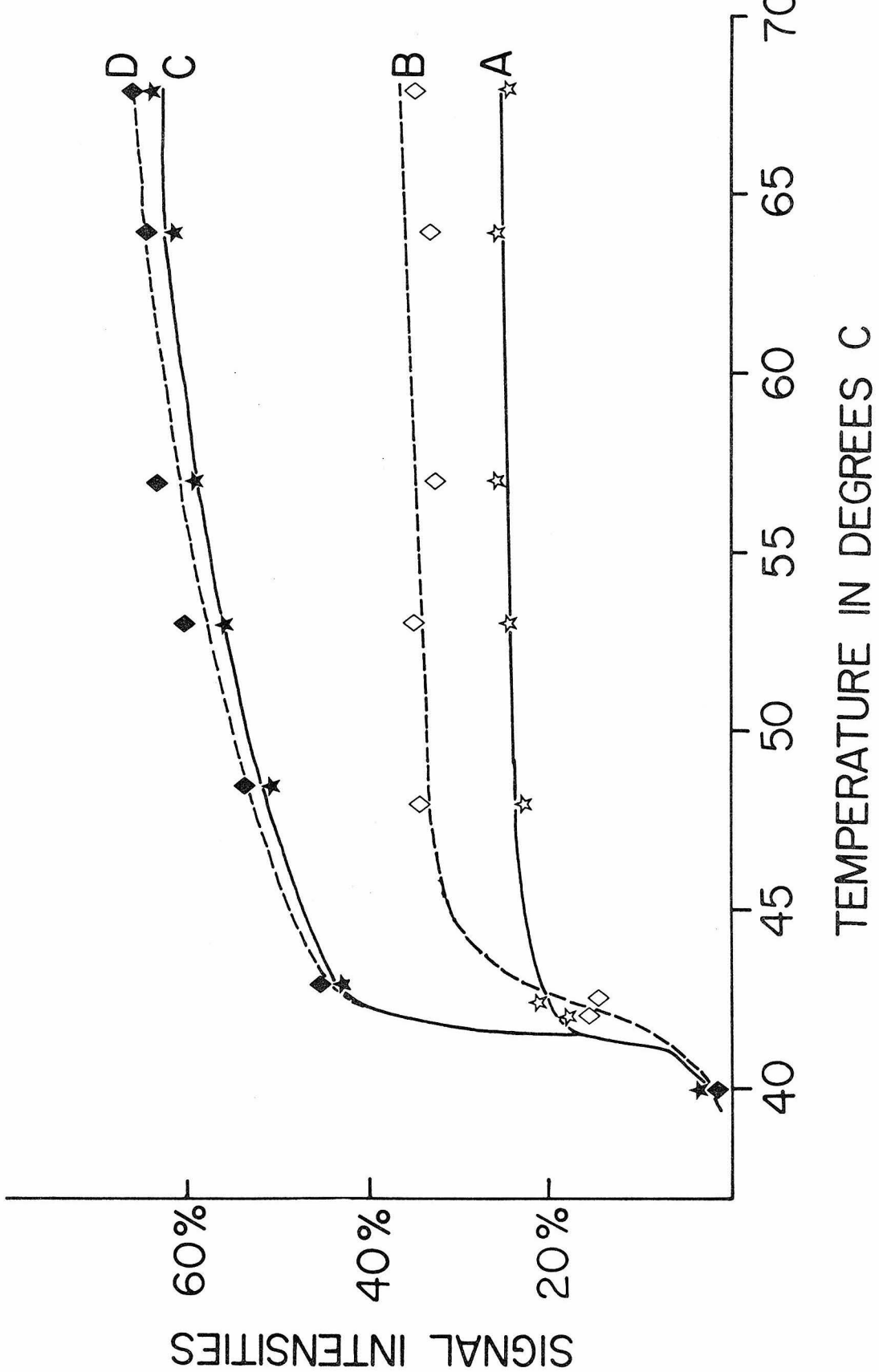
Spin-spin relaxation times were measured with a 54 MHz pulse nmr spectrometer as mentioned in the last chapter.

3.4 Results and Discussion

The effect of alamethicin on the crystalline $\rightleftarrows$ liquid crystalline phase transition of dipalmitoyllecithin bilayers has been followed using the DFT nmr method by monitoring the intensities of the two methyl signals as a function of temperature. Figure 5 summarizes the intensities of the choline and terminal methyl proton signals as a function of temperature for lecithin multilayers containing zero and 1% alamethicin. Below the thermal phase transition, the molecular motions of the phospholipid molecules are slow and seriously restricted, and as a consequence no high-resolution features appear in the DFT pmr spectrum. In

FIGURE 5

Variations of the intensities of choline and terminal methyl proton signals of unsonicated dipalmitoyllecithin multilayers with temperature: (A) choline methyl protons without alamethicin; (B) choline methyl protons in the presence of 1% alamethicin; (C) terminal methyl protons without alamethicin; (D) terminal methyl protons in the presence of 1% alamethicin.


SIGNAL INTENSITIES
60%
40%
20%
40
45
50
55
60
65
70
TEMPERATURE IN DEGREES C
A
B
C
D

the liquid crystalline phase, however, considerable molecular motion is present and the methyl resonances emerge, their intensities determined by the degree of motional restriction. It was noted that the addition of 1% alamethicin does not produce significant changes in the phase transition temperature, except that the temperature range of the transition becomes somewhat broadened. The addition of alamethicin does affect the choline methyl signal intensity, however. In the presence of 1% alamethicin, the intensity of the choline signal becomes 30-35% of the total expected intensity as compared to only 20-25% in the absence of the polypeptide. By contrast, upon addition of 1% alamethicin, the terminal methyl proton resonance exhibits no noticeable change in either intensity or line width.

The effect of alamethicin on the transverse relaxation time (T_2) of the bulk nmr signal is summarized in Fig. 6. Below the phase transition, the bulk nmr signal is characterized by a T_2 of less than 30 μsec; above the phase transition temperature this relaxation time is increased to 110 μsec or more. Addition of 1% alamethicin reveals no noticeable effect on the apparent T_2 above the thermal phase transition. The alamethicin was found to broaden the transition temperature range by some 1-2° in accordance with the DFT observation reported earlier in this chapter.

The above observations strongly suggest that alamethicin interacts with dipalmitoyl lecithin bilayer membranes principally at or near the bilayer-water interface. Although our experiments do not allow us to ascertain the various degrees to which the mobility of each individual

FIGURE 6

Temperature dependence of the spin-spin relaxation time of the bulk nmr signal from dipalmitoyllecithin multilayers in the absence and presence of alamethicin: solid line, no alamethicin; dashed line, with 1% alamethicin.

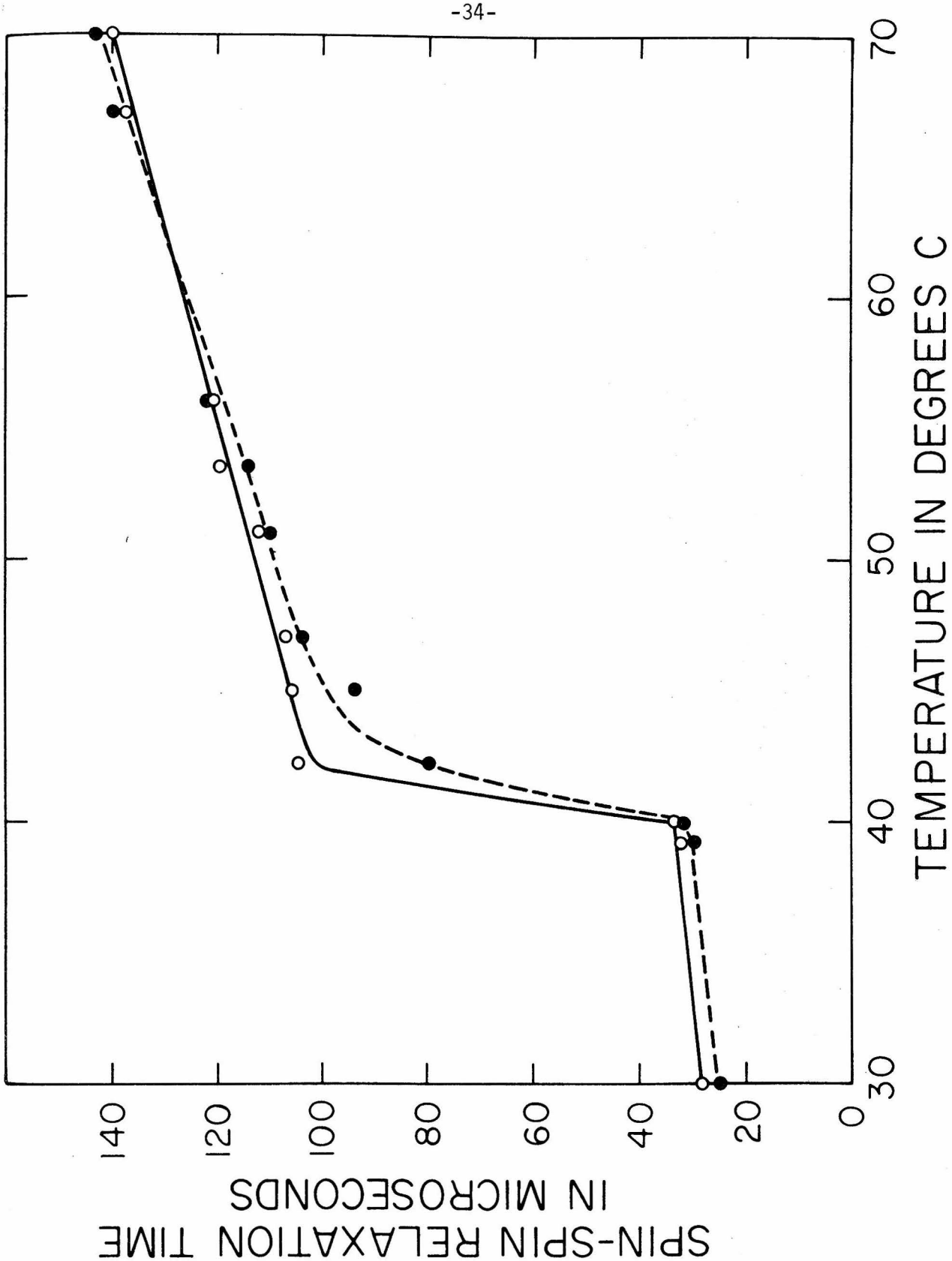

SPIN-SPIN RELAXATION TIME
IN MICROSECONDS
0
20
40
60
80
100
120
140
30
40
50
60
70
TEMPERATURE IN DEGREES C

TABLE I. Effect of Alamethicin on the Transverse Relaxation Time of the Bulk NMR Signal of Dipalmitoyllecithin Bilayers at Various Temperatures

	T_2 μsec	
Temperature °C	Sample Containing no Alamethicin	Sample Containing 1% Alamethicin
30	28	26
39	34	30
40	36	32
42	106	80
45	107	94
47	108	106
56	124	124
60	124	126
67	138	141
70	140	144

Chapter IV

INTERACTIONS OF ALAMETHICIN WITH SMALL SONICATED LECITHIN BILAYER VESICLES

4.1 Introduction

The effect of alamethicin on sonicated bilayer vesicles has been studied using conventional Fourier transform and continuous wave nmr techniques. A typical pmr spectrum of small sonicated vesicles consists of two partially resolved choline signals located at about 3.21 ppm, a somewhat broader fatty acid chain signal centered at 1.2 ppm and a terminal methyl proton signal superimposed on the latter. The spectral features of these resonances differ from those of unsonicated multilayers in both intensity and linewidth. Unlike the broad multilayer signals which usually have linewidths of 100 Hz (choline methyl signal) to over 3000 Hz (methylene chain signal), small vesicle pmr linewidths are much narrower ($\sim$ 5 Hz for choline methyl protons and $\sim$ 30 Hz for methylene chain protons). The intensities of these sharp resonances normally account for 80% or more of the stoichiometric amount of protons present in the sample, in contrast to the situation in multilayers in which only about 25% (choline methyl signal) and 50% (terminal methyl signal) of the total expected intensities are observable. Apparently the large surface curvature of small sonicated vesicles makes for significantly looser packing of the phospholipid molecules; this accounts for these strikingly different pmr spectral characteristics of sonicated and unsonicated bilayer systems.

Even the difference in surface curvatures on the outer and inner halves of the bilayer vesicle results in different magnetic environment for the choline methyl protons.

As explained in Chapter I, small bilayer vesicles, being thermodynamically less stable than multilayers, have a tendency to undergo vesicle-vesicle fusion in order to minimize their free energy. This tendency is more apparent when a surface active species such as alamethicin is present in the system as a nucleation center, as we observed in the present work by negative staining electron microscopy experiments.

4.2 Sample Preparation

Sonicated bilayer vesicles were prepared using a Branson sonifier equipped with a microtip. A known quantity of purified lecithin was weighed into a centrifuge tube, to which D_2O (99.8% ^2H) containing 2mM phosphate (sodium salt) at pD 7.4 was added to give a suspension of about 50 mg of lecithin per ml D_2O. Sonication at level 4 for about 15 minutes produced a nearly transparent, colorless solution. This was centrifuged at 12,000 rpm in a Superspeed RC-2 centrifuge for 40 minutes to remove residual multilayers and large vesicles from the smaller vesicles suspended in the supernatant. The latter was used in all subsequent nmr and EM experiments.

In order to avoid possible degradation of the alamethicin, the antibiotic was never sonicated together with the lecithin in our experiments. Instead, it was first weighed out in a 1 ml volumetric flask, dissolved in D_2O containing 2 mM phosphate at pD 7.4, and a

known volume of this solution was added to a sonicated preparation of lecithin vesicles. This resultant mixture was then equilibrated using a vortex mixer for less than a minute and nmr and EM experiments on this solution were undertaken immediately.

Since sonication results in some change in the lecithin concentrations, final calibrations of the lecithin concentrations in small vesicles were performed using the dry weight method. After the addition of alamethicin, lecithin and alamethicin contrations in the final mixture were calculated assuming that their partial molal volumes were not changed upon mixing. This is a reasonable assumption in our work, since the volumes of alamethicin solutions used were usually only on the order of a few hundredths of a millimeter vs a total volume of 4 ml and the concentration of alamethicin was low ($\sim 10^{-4}$M).

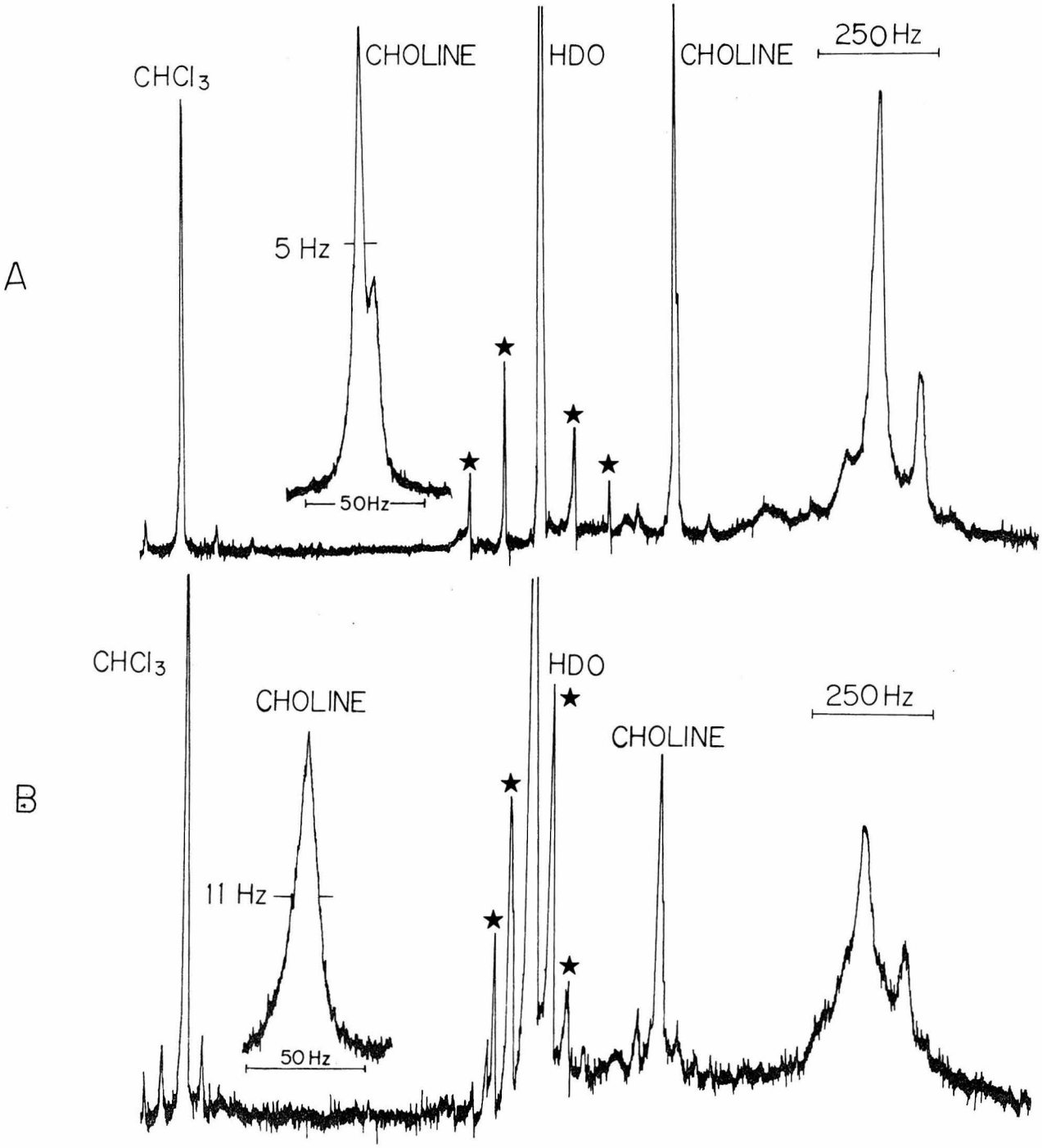
4.3 Results and Discussion

Freshly prepared solutions of small sonicated vesicles are colorless, nearly transparent and homogeneous. Upon the addition of alamethicin, however, cloudiness developed in the sample in 10-15 minutes, depending on temperature and the relative amounts of lecithin and alamethicin in the solution. This change in the turbidity immediately suggests some alteration in the state of aggregation and/or size distribution of the bilayer vesicles. This conclusion is indeed substantiated in this piece of work.

Representative pmr spectra of sonicated vesicles in the absence and presence of alamethicin are shown in Fig. 7. It is

FIGURE 7

PMR spectra (220 MHz) of sonicated dipalmitoyllecithin bilayer vesicles at 48°. Spectrum A is for a sample containing no alamethicin and spectrum B is for a sample containing 0.3% alamethicin. Note that the choline signal is also shown in expanded scale in both spectra. Asterisks denote spinning side bands associated with the HOD signal.


A
CHCl3
CHOLINE
HDO
CHOLINE
250 Hz
5 Hz
50Hz
B
CHCl3
CHOLINE
HDO
250 Hz
CHOLINE
11 Hz
50 Hz

evident that alamethicin produces line broadening as well as intensity reduction of the entire pmr spectrum. This variation in the intensities of the fatty acid chain and choline methyl proton signals upon the addition of alamethicin is explicitly depicted as a function of temperature in Fig. 8. It is interesting to note that the observed temperature variation of the intensities for these pmr signals very nearly resemble those previously reported by Sheetz and Chan (1972) for bilayer vesicles of diameter 1000Å.

Electron microscopy studies confirmed that the size distribution of the vesicles has indeed been altered with addition of alamethicin. Figure 9 shows representative electron micrographs of lecithin vesicle preparations with and without the addition of alamethicin. The variation of the size distribution of lecithin vesicles by weight in various diameter ranges as a function of alamethicin concentration is summarized in Table II and also graphically presented in Fig. 10. From these results it is obvious that the addition of alamethicin has promoted fusion of vesicles. The shape of the size distribution function is alamethicin-concentration-dependent as expected. Although 0.01% of alamethicin affects the size distribution only minimally, 0.4% alamethicin significantly broadens out the size distribution. These electron microscopic results closely parallel the nmr results cited earlier. The nmr experiments showed that although the intensities of the various proton signals are still around 90% of their expected intensities in the presence of 0.01% alamethicin, these pmr intensities are reduced by some 50% when 0.4% alamethicin is present (Fig. 11).

FIGURE 8

Temperature dependence of the choline methyl and fatty acid chain proton signal intensities for sonicated lecithin vesicles and vesicles incubated with 0.3% alamethicin: (A) choline methyl protons; (B) fatty acid protons. In both A and B, the solid lines give the temperature dependence in the absence of alamethicin and the dashed lines refer to the results obtained in the presence of 0.3% alamethicin.

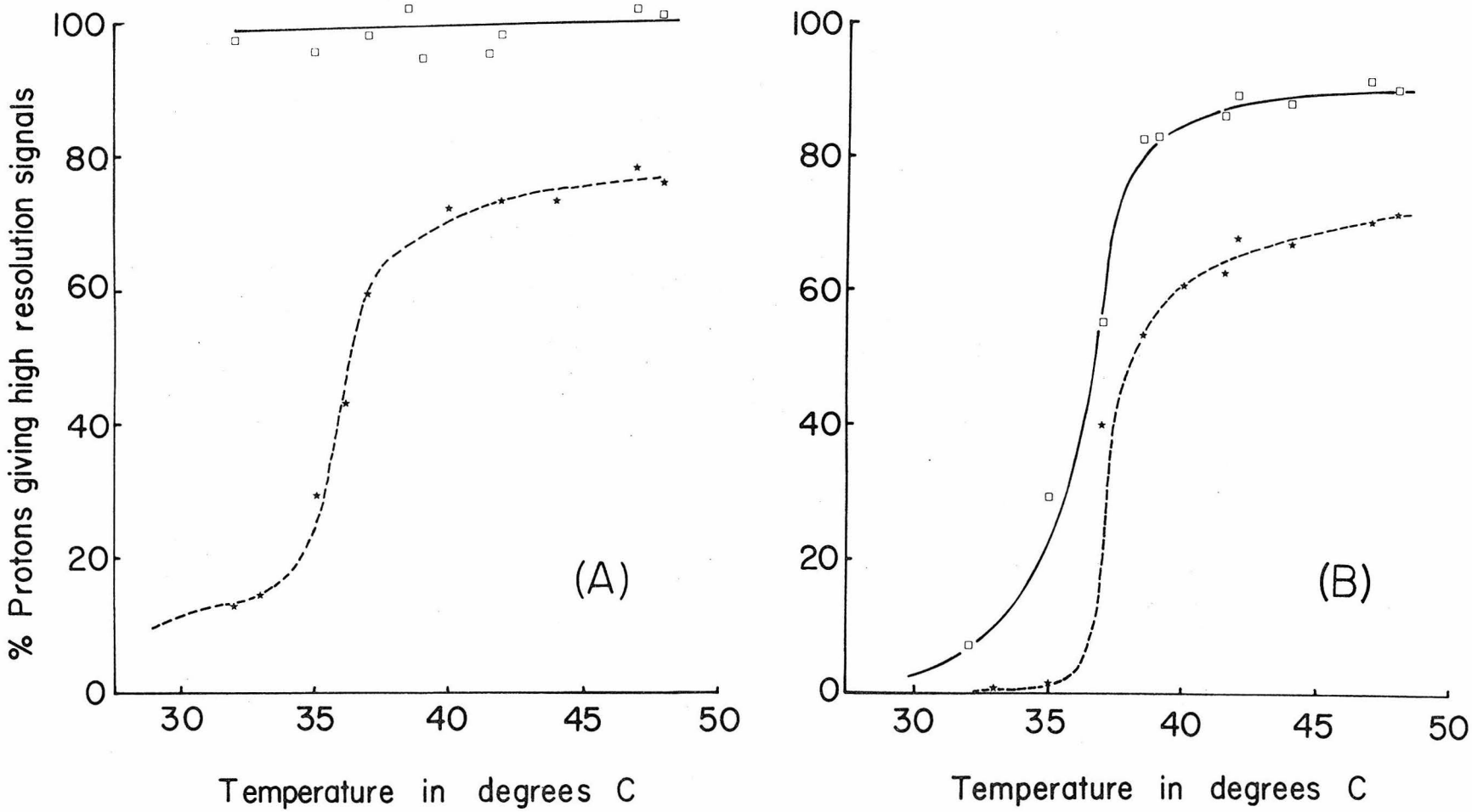
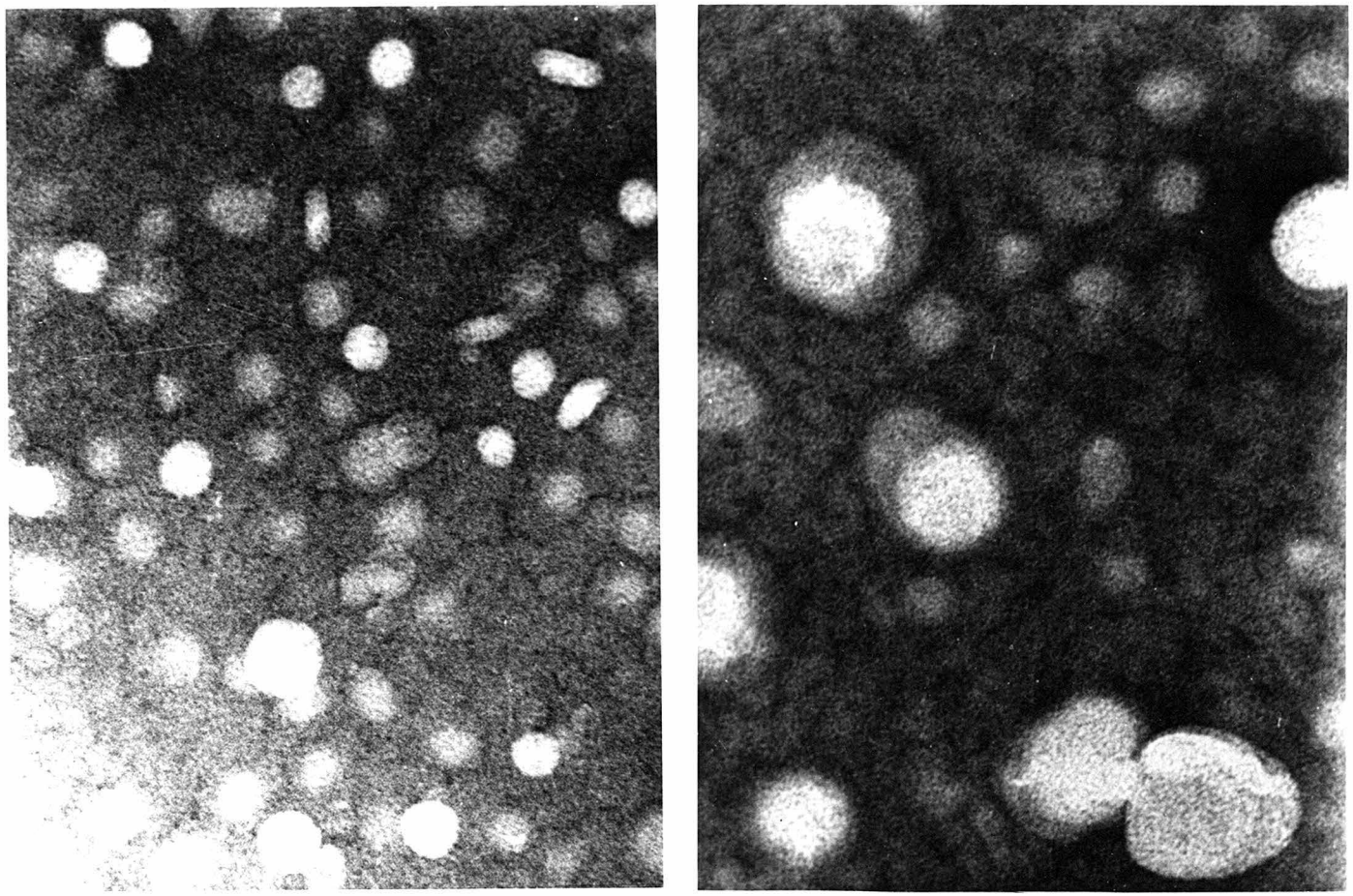

% Protons giving high resolution signals
100
80
60
40
20
0
30
35
40
45
50
Temperature in degrees C
(A)
(B)

FIGURE 9

Representative micrographs for lecithin vesicle preparations.



REPRESENTATIVE MICROGRAPHS OF LECITHIN VESICLES

Left No alamethicin added
Right Lecithin / alamethicin = 250

1000 Å

FIGURE 10

Cumulative frequency function of vesicle sizes: (A) sample containing no alamethicin; (B) (dashed line) 0.01% alamethicin; (C) 0.4% alamethicin.

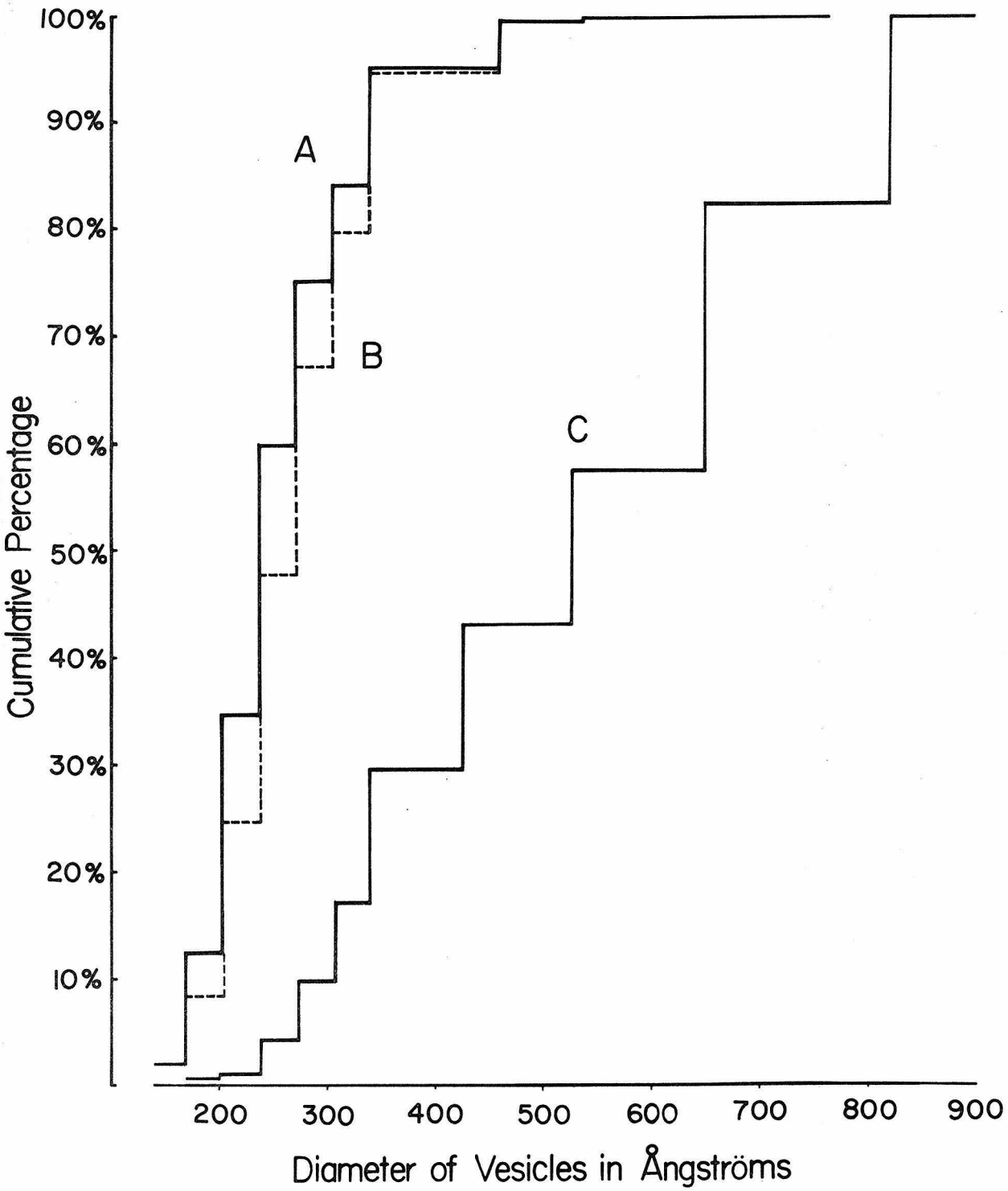
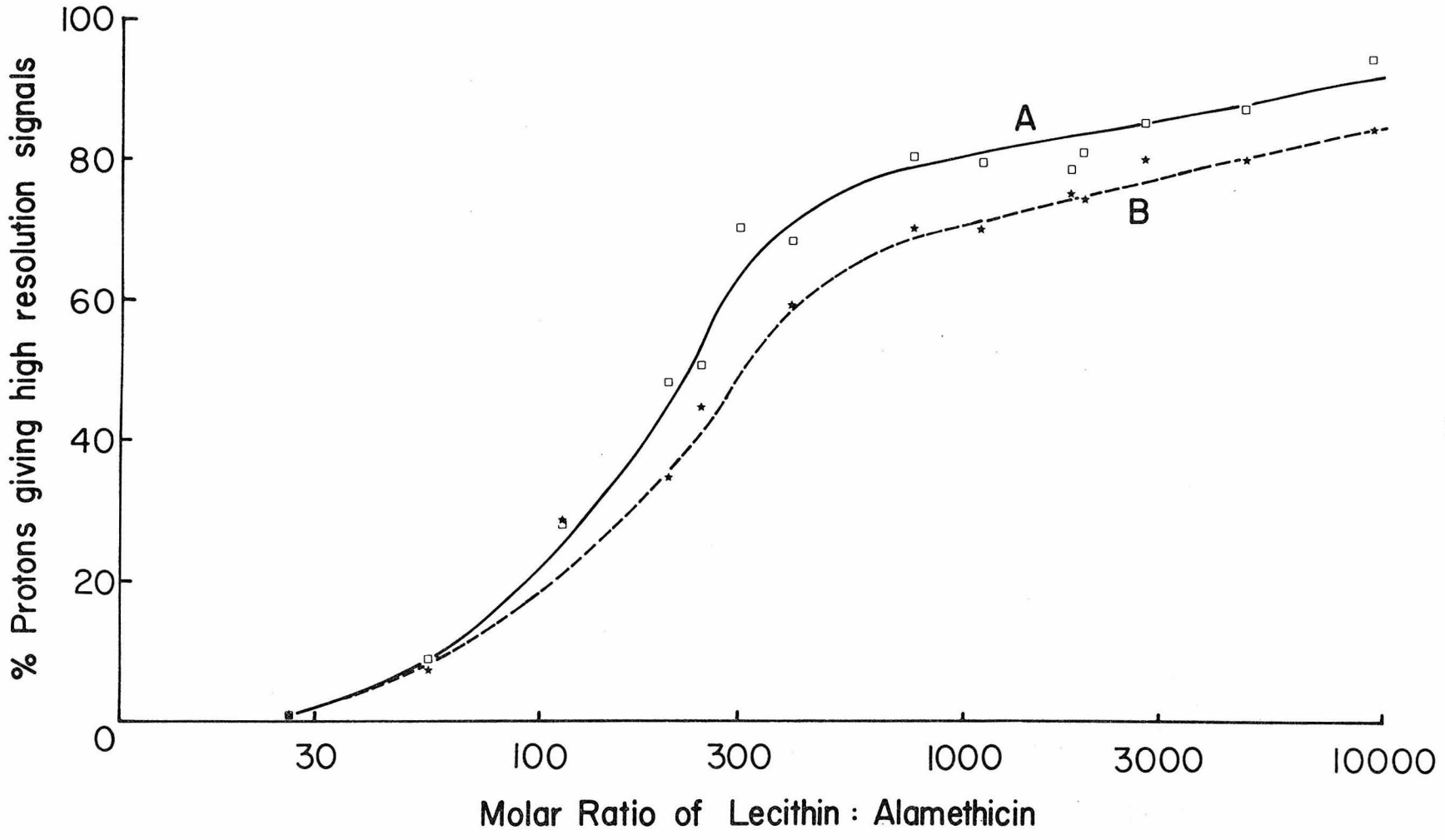

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
Cumulative Percentage
A
B
C
200
300
400
500
600
700
800
900
Diameter of Vesicles in Ångströms

FIGURE 11

Effect of alamethicin concentration on the pmr signal intensities of sonicated lecithin vesicles: (A) choline methyl protons; (B) fatty acid chain protons.


% Protons giving high resolution signals
100
80
60
40
20
0
A
B
30
100
300
1000
3000
10000
Molar Ratio of Lecithin : Alamethicin

A comparison of the temperature dependencies of the choline methyl proton intensities of vesicles containing alamethicin (Fig. 8A) with that of 1000Å vesicles does reveal an important difference. In normal large vesicles 1000Å in diameter, the intensity of the choline methyl signal was previously reported (Sheetz and Chan, 1972) to approach 100% of the stoichiometric proton concentration when the temperature is above 40°. In the alamethicin-treated vesicle samples only about 80% of the expected choline methyl pmr intensity was observed even at 48°. This difference suggests that there exist specific interactions of the antibiotic molecule with the lecithin bilayer and that these interactions result in immobilization of some of the choline methyl groups at or near the vicinity of the adsorbed oligopeptide. This immobilization of the polar head groups in the case of single wall bilayer vesicles can be contrasted to the loosening up of the structure near the polar head group region in the case of flat multilayers. This important difference between the behavior of curved and flat bilayers to the effect of alamethicin is not unexpected in view of the difference in the molecular packing between these two types of bilayers.

The above findings on alamethicin mediated fusion of vesicles constitute further support for the notion that this antibiotic is highly surface active. Presumably when this antibiotic is adsorbed on the surface of a bilayer vesicle it can act as a nucleating center for further coagulation and subsequent fusion of the vesicles. In the next chapter we shall investigate the nature of this fusion process in great detail with the help of europium (III) ions as an nmr shift reagent.

Chapter V

ALAMETHICIN-MEDIATED FUSION OF LECITHIN VESICLES

5.1 Introduction

The nmr and EM results reported in the last chapter can readily be accounted for by the alamethicin-induced fusion, as well as by interactions of the polypeptide with the bilayer in the vicinity of the water-bilayer interface. The observed increase in average vesicle size cannot be explained in terms of alamethicin incorporated into the hydrophobic core of the bilayer membrane, since, in the absence of vesicle fusion, this process could only lead to an increase of vesicle volume of no more than 1% at the highest concentration of alamethicin used in this work.

As explained previously, because small vesicles possess high surface free energy by virtue of their large curvature, they would undergo rearrangements in their molecular packing to attain a state of greater stability once this opportunity is present. In this chapter we shall first put forward a model for this process of surfactant-induced vesicle fusion, and then describe a series of experiments which indeed establishes the validity of this model.

5.2 Model for Alamethicin-Induced Vesicle Fusion

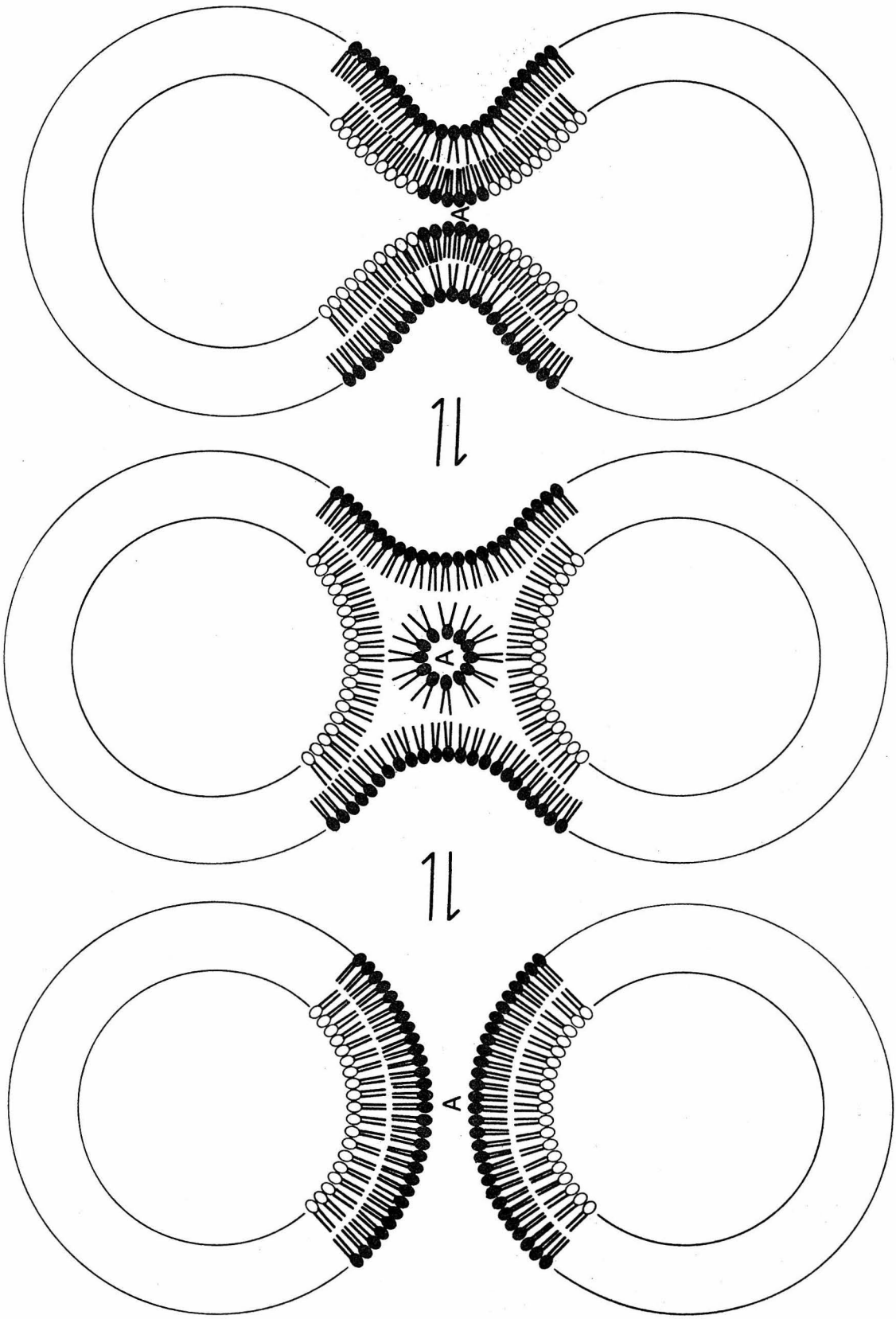
Vesicles can fuse when, as a result of thermal motion, they approach each other sufficiently closely to overcome the repulsive interactions between their electrical double layer potentials. Van der Waals interaction between the polar head groups of the phospholipid

molecules from different vesicles can cause transient rearrangements in the local structure with the resultant formation of an inverted micelle. That this latter event must occur is evident from the fact that translocation of phospholipid molecules must accompany the fusion of two or more 300Å diameter vesicles. Simple arithmetic shows that about 12% of the phospholipid molecules on the outer half of the bilayer vesicle must be transferred to the inner half during the fusion of several 300Å vesicles to form an 800Å vesicle. Rothman and Dawidowicz (1975) have recently shown, in their study of lecithin exchange using red blood cell ghost and ^{3}H-labelled phospholipid bilayer vesicles, that no detectable phospholipid flip-flop was observed even in five days. The half life of a flip-flop was estimated to be longer than 11 days. Such a slow rate of lipid transit between inside and outside (if any occurs at all) definitely cannot account for the rather rapid transfer of lipid molecules (~12% in less than half an hour) in the presence of alamethicin-induced fusion.

A surface active molecule such as alamethicin might be expected to serve as a catalyst for such fusion. Fusion can be initiated, for example, by clusters of alamethicin molecules located on the vesicles' surfaces. As such clusters readily penetrate the thickness of the electrical double layer of these vesicles, they can serve as nucleation centers for micelle formation. In other words, the clusters act as a detergent. A possible sequence of alamethicin-mediated vesicle fusion is schematically represented in Fig. 12. Termination of this process occurs when the alamethicin concentration in the extravesicular aqueous medium has been reduced to a sufficiently low level as a result of this endocytosis process, and/or when the vesicles have grown to a sufficiently large size that further fusion results in no

FIGURE 12

Schematic representation of the sequence of events for the alamethicin-mediated fusion of vesicles. A = alamethicin aggregate (not to scale).


A
1l
A
1l
A

substantial gain in stability. As one might expect, after fusion the alamethicin cluster may find itself trapped within the fused vesicle. The fact that the total choline pmr signal intensity approaches zero as the alamethicin concentration is increased (Fig. 11) appears to provide the first piece of evidence in support of this model, for it is unlikely that total annihilation of choline signal could occur without some kind of physical interaction of the alamethicin with the polar head groups on the inner half of the bilayer. In the mind of the critics, of course, the possibility of strong coupling between the two halves of the bilayer remains. In the following section we shall describe a series of experiments which provides confirming evidence for the incorporation of alamethicin inside the intravesicular space as a result of vesicle fusion, and rules out the possibility of transmembrane coupling.

5.3 The Use of Paramagnetic Shift Reagents

Our rationale for this work was that the translocation of alamethicin into the intravesicular solution could be ascertained by studying the effect of externally added alamethicin on the choline head groups located on the inner and outer halves of the bilayer separately. In the pmr spectrum of lecithin bilayer vesicles, the choline methyl protons give rise to two partially resolved signals at $\sim$ 3.2 ppm due to the different surface curvatures of the outside and inside surface of the bilayer unit. Although the small chemical shift difference between these two overlapping signals ($\sim$ 5 Hz at 51.7 kgauss depending on temperature) does complicate the analysis of the spectral data, complete

resolution of these choline signals is possible when the experiments are carried out with certain paramagnetic shift reagents in the extravesicular medium as demonstrated by Bystrov and co-workers(Bystrov et al., 1971; Shapiro et al., 1975). More recently, Levine et al. (1973) and Kostelnik and Castellano (1972) showed that by using appropriate quantities of europium (III) ions, it is, in fact, possible to resolve the two choline methyl signals completely without significantly altering their linewidths. We used this technique in our work.

5.4 Sample Preparation

Sonicated bilayer vesicles (average diameter 250-300Å) were prepared using a Branson sonifier. A known quantity of purified lecithin was weighed into a centrifuge tube, to which D_2O containing 2 mM phosphate (sodium salt), 0.1 M NaCl and 0.05 M $La(NO_3)_3$ at pD 7.4 was added to give a suspension of about 50 mg lecithin per ml solvent. The mixture was sonicated and centrifuged as described in Chapter IV.

Alamethicin was weighed into a 1 ml volumetric flask, dissolved in appropriate amount of D_2O containing 2 mM phosphate, 0.1 M NaCl and 0.05 M $La(NO_3)_3$ at pD 7.4. Europium nitrate and sodium chloride were weighed into a 25 ml volumetric flask, to which D_2O containing 2 mM phosphate at pD 7.4 was added so that the resulting solution was isotonic with the vesicle solution.

Three sets of experiments were performed. In one series, known quantities of alamethicin and vesicle solutions were mixed and incubated at 70° for 20 minutes. To the incubated mixture a measured amount of the europium nitrate-sodium chloride solution was added. The

nmr and EM experiments followed immediately.

In another series of experiments, the europium nitrate-sodium chloride solution was mixed with the sonicated vesicle solution before the addition of alamethicin. After alamethicin was added, the time course of the pmr spectrum of the inner and outer choline groups was followed using FT-nmr spectroscopy.

Finally, the effect of alamethicin on a lecithin vesicle suspension sonicated in the presence of europium (III) ions was also studied. To the sonicated mixture a small quantity of alamethicin ($\sim$ 0.01%) was added. After fusion had subsided, the mixture was dialysed at room temperature against an isotonic NaCl-$La(NO_3)_3$ solution for four hours. NMR and EM experiments were then performed on the dialysed mixture.

5.5 Results and Discussion

5.5.1 Determination of Relative Lipid Concentrations on the Two Halves of a Bilayer Vesicle. It has been established that the surface curvature of a vesicle has a profound effect on the molecular packing of a small bilayer vesicle (Sheetz and Chan, 1972). A vesicle of diameter 300Å, for example, has approximately 100Å, 125Å and 150Å as radii of curvature for its inner, median, and outer surfaces respectively (see Fig. 13 for definitions of these surfaces). Since the difference between them is rather substantial, arrangement of the lipid molecules would be expected to be non-identical for the two halves of the bilayer vesicle. Do these monolayers partition the lipid molecules according to the ratio of their surface areas or to the ratio of their respective volumes? This question must be answered before any meaningful

determination of pmr signal intensities can be performed in this chapter. We have prepared DPL vesicles of different sizes by varying the sonifying power and length of sonication time. Europium (III) ions were added to the external solution to resolve the two choline methyl signals. Relative intensities of these two signals were determined as a function of average vesicle size (vesicle size determined by negative staining electron microscopy). The results are depicted in Figs. 13 and 14. Theoretical curves for ratios of outer/inner surface areas, median/inner surface areas and outer/inner lipid volumes were computed and are present in the same figure for comparison. It is obvious that the relative amount of lipid in the two halves of a vesicle may be represented either by curve B or curve C, these two curves being not substantially different from each other. Curve A is significantly different from the experimental results. Therefore in the following section we shall calculate the expected lipid concentrations of the inner and outer halves of the bilayer vesicle according to the ratio of their respective volumes.

5.5.2 Effect of Alamethicin-Induced Fusion on the Inner and Outer Choline Head Groups. The effect of alamethicin-induced fusion of vesicles on the inner and outer choline methyl groups can be ascertained separately if appropriate amounts of europium (III) ions are added to the solution after fusion has taken place. The added europium (III) ions shift the outer choline methyl signal to higher fields in the pmr spectrum, thus permitting the two choline signals to be monitored separately.

FIGURE 13

Variation of the ratio of outer:inner lipid concentrations as a function of vesicle diameter. Curve A: outer surface area vs inner surface area. Curve B: median surface area vs inner surface area. Curve C: outer lipid volume vs inner lipid volume. Curve D (solid line): experimentally determined values.

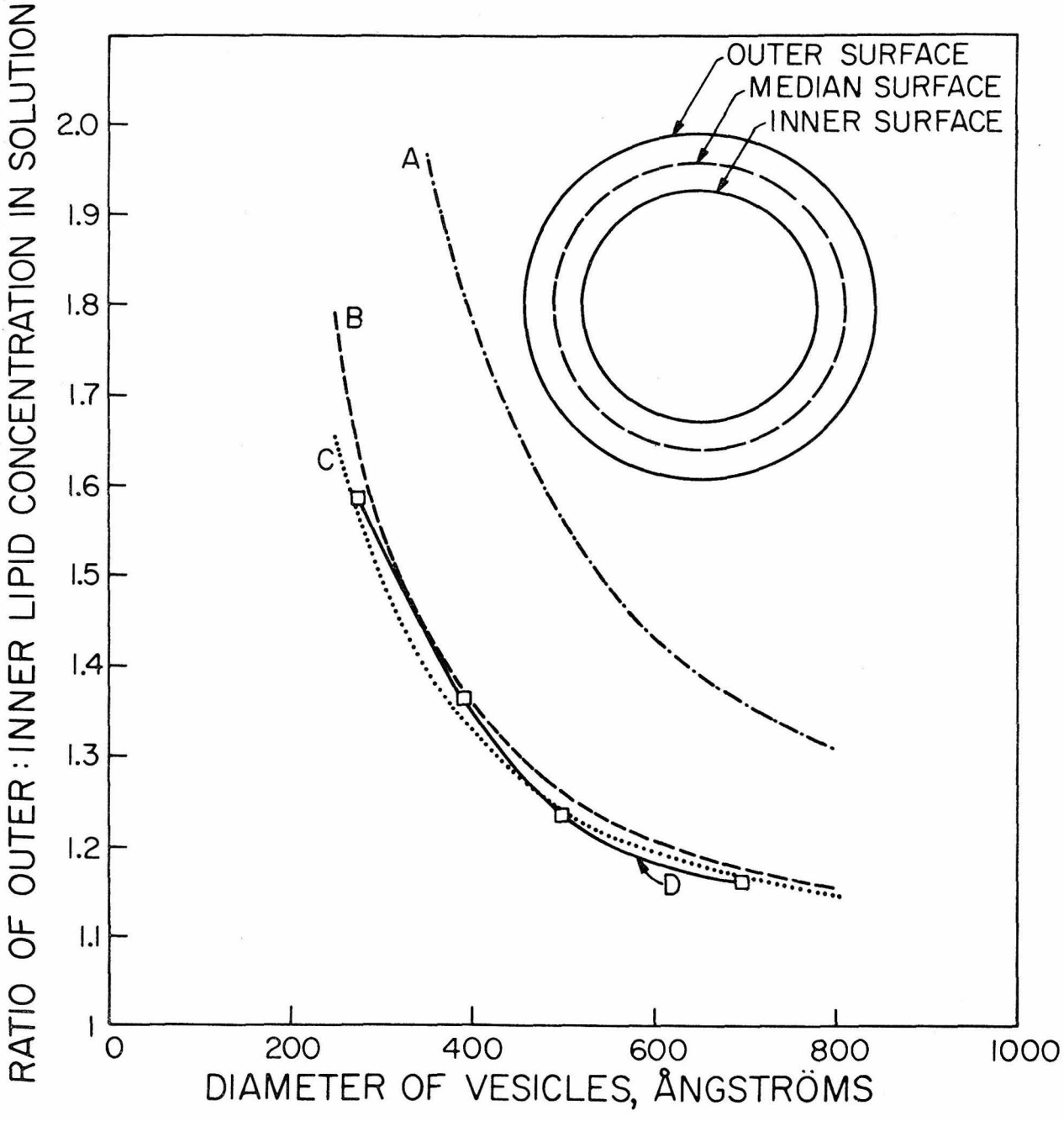
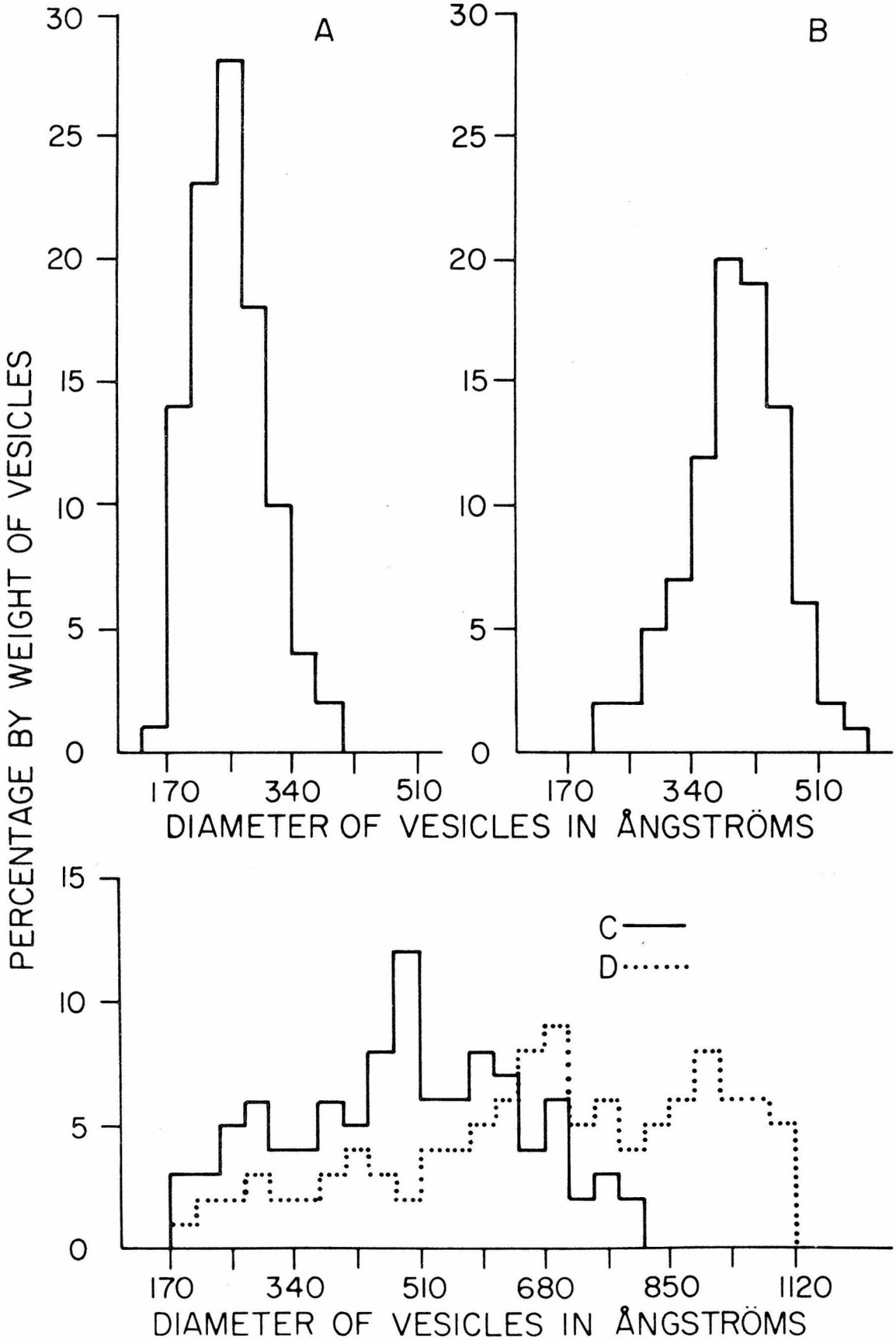

RATIO OF OUTER : INNER LIPID CONCENTRATION IN SOLUTION
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0
200
400
600
800
1000
DIAMETER OF VESICLES, ÅNGSTRÖMS
OUTER SURFACE
MEDIAN SURFACE
INNER SURFACE
A
B
C
D

FIGURE 14

Size distributions of vesicles for the four data points in Curve D of Fig. 13.


A
B
30
25
20
15
10
5
0
170
340
510
PERCENTAGE BY WEIGHT OF VESICLES
DIAMETER OF VESICLES IN ÅNGSTRÖMS
C
D
680
850
1120

The effect of alamethicin and its associated induced-fusion on the intensities and linewidths of the inner and outer choline signals are summarized in Table III. The observed variation of the signal intensities with alamethicin concentrations is also graphically presented in Fig. 15. Since variations in the size distribution of the vesicles during the fusion process change the stoichiometric proportions of lipid molecules in the two halves of the bilayer, proper interpretation of this intensity data can only be made after transfer of lipid from the outer to the inner half of the bilayer has been taken into account. Accordingly, these intensities are expressed in terms of the percentage of lipid expected for each half of the bilayer for the size distribution of bilayer vesicles determined (by electron microscopy) at the time the nmr measurements were performed. Both the inner and the outer choline signals exhibit a decrease in intensity as the alamethicin concentration in the sample increases, in accordance with results reported earlier (Hauser et al., 1970; Lau and Chan, 1974; also see Chapter IV of this thesis). We note, however, that despite their location supposedly shielded from the external solution, the choline methyl protons from the inner half of the bilayer actually manifest a somewhat greater reduction in their pmr signal intensity. This provides evidence that some of the antibiotic molecules become incorporated into the intravesicular space during

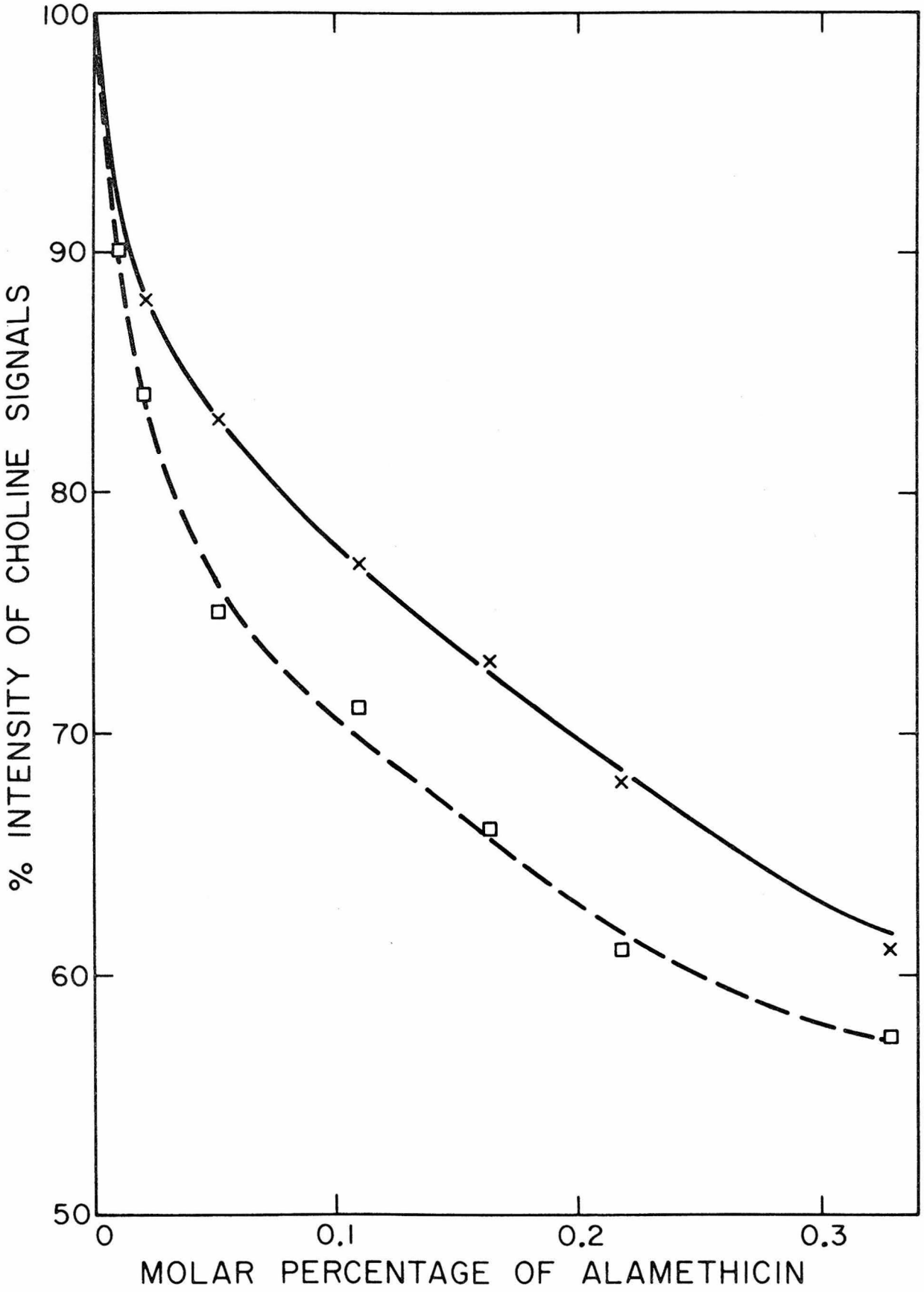
TABLE III. Variations in intensity and linewidth of the inner and outer choline signals in the presence of alamethicin*

	Outer choline		Inner choline	
Alamethicin (molar %)	Linewidth (Hz)	Intensity (%)	Linewidth (Hz)	Intensity (%)
No alamethicin	5.6	100	4.6	100
0.02	6.4	88	5.4	84
0.06	7.0	83	6.0	75
0.11	8.6	77	7.8	71
0.16	∿9	73	∿10	66
0.22	∿10	68	∿10.5	61

*Europium (III) ions (7 mM) added to the extravesicular solution only. The vesicles were fused for 20 min at 70°. Intensities are expressed in terms of lipid concentrations expected for each half of the bilayer for the size distribution of vesicles determined at the time the NMR experiments were performed.

FIGURE 15

Variations of the intensities of the inner and outer choline methyl signals of sonicated dipalmitoyllecithin bilayer vesicles as a function of alamethicin concentration. Solid line is for choline signal from the outer half of the bilayer vesicle and dashed line is for the inner choline signal. Data have been corrected for changes in the stoichiometric lipid concentrations as a result of vesicle fusion. Alamethicin concentration is expressed as molar percentage of the phospholipid in the dispersion.


100
90
80
70
60
50
% INTENSITY OF CHOLINE SIGNALS
0
0.1
0.2
0.3
MOLAR PERCENTAGE OF ALAMETHICIN

the fusion process.

There are two possible reasons for the choline signals' intensity loss when alamethicin is added to these lecithin vesicle solutions. First, the alamethicin-induced fusion increases the average vesicle size, resulting in slower rotational tumbling of the bilayer units as well as less effective averaging of the dipolar broadening by surface diffusion and molecular tumbling of the lipid molecules. Secondly, alamethicin interacts physically with the polar head group region of the lecithin bilayer, with a resultant decrease in the flexibility and mobility of the choline head groups. In the present experiment, both factors contribute to the apparent intensity anomaly, since it has been shown that the motion of these choline head groups is essentially unrestricted and rapid even for large vesicles approximately 1000Å in diameter at temperatures over 40° (Lichtenberg et al., 1975).

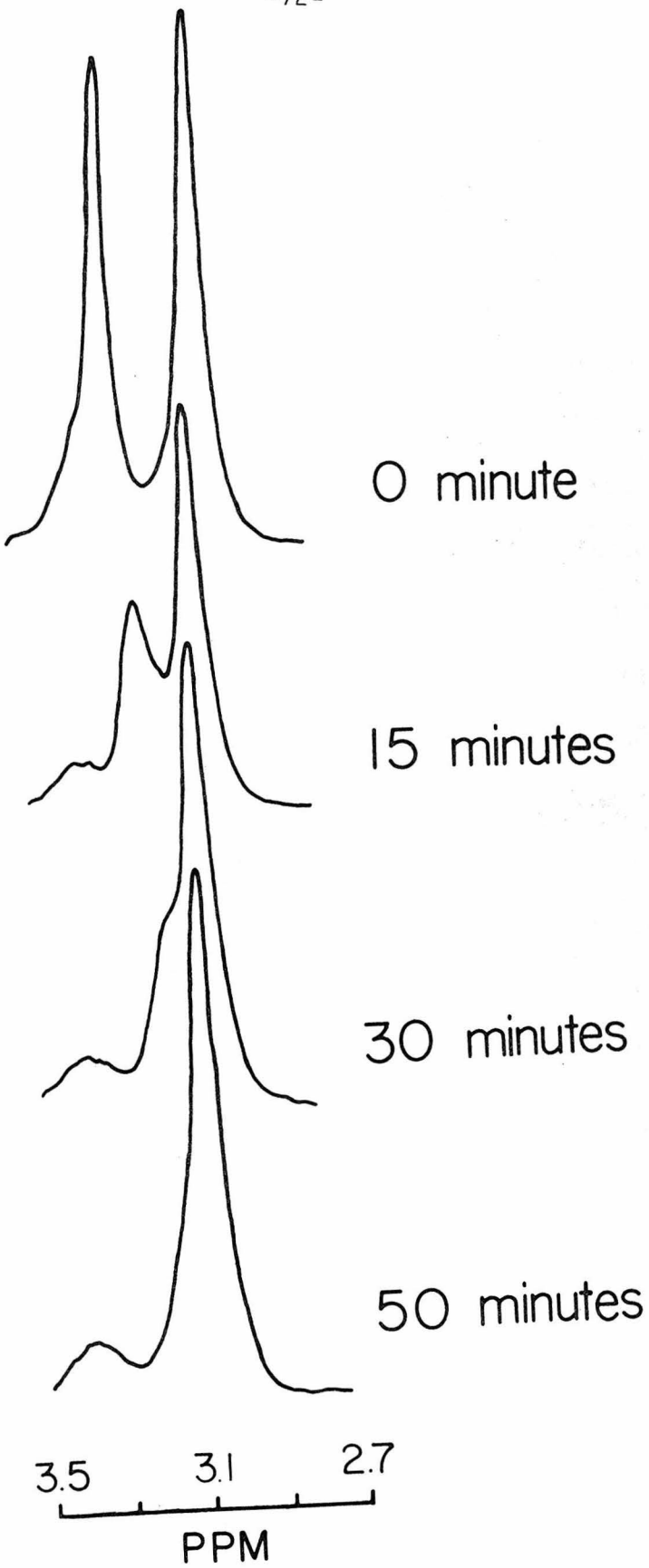
5.5.3 Translocation of Paramagnetic Ions across the Bilayer Membrane during Alamethicin-Mediated Vesicle Fusion. In the previous section we have presented evidence for the entrapment of alamethicin molecules into the intravesicular volume in consequence of the antibiotic-mediated fusion. Since alamethicin is negatively charged we expect, as a corollary, that counterions will be translocated as well. This point could be ascertained if, for example,

europium (III) ions were present in the extravesicular medium prior to the onset of fusion. A series of experiments was therefore carried out under these conditions.

The results of this study are summarized in Fig. 16, where the choline spectrum is displayed as a function of time after the addition of alamethicin to the solution. These data show a continuous upfield shift of the inner choline signal relative to the outer choline resonance accompanied by a concomitant intensity reduction of both signals as the fusion process progresses until it finally merges completely with the outer choline signal some 50 minutes after the addition of alamethicin. Since the intravesicular solution contained no europium (III) ion originally, the observed upfield shift of the inner choline signal must arise either from the translocation of europium (III) ions during fusion, or must be the result of europium ion transportation through alamethicin pores formed in the bilayer membrane, or must be the result of passive permeation of europium (III) ions through the bilayer. The last possibility can be readily dismissed, since in a separate experiment (A. Lau, unpublished experiment) we have shown that lecithin bilayers are impermeable to europium (III) ions in the absence of alamethicin even after incubation of the vesicles at 70° for over three hours. In the next section, we shall also rule out the possibility of alamethicin pores. Hence the rapid translocation of europium (III) ions must be associated with the fusion

FIGURE 16

Time evolution of the 100 MHz pmr spectra of the choline methyl protons when the alamethicin mediated fusion of vesicles is carried out in the presence of 7×10^{-3}M europium (III) ions in the extravesicular solution. The antibiotic is added at 0 minute. Incubation temperature is 70°. Alamethicin concentration in the sample is 0.2%. Chemical shifts are measured downfield from an external TMS signal without making bulk magnetic susceptibility corrections. The broad resonance at 3.4 ppm is due to the $Me_3N\text{-}\underline{CH_2}$ protons.


0 minute
15 minutes
30 minutes
50 minutes
3.5
3.1
2.7
PPM

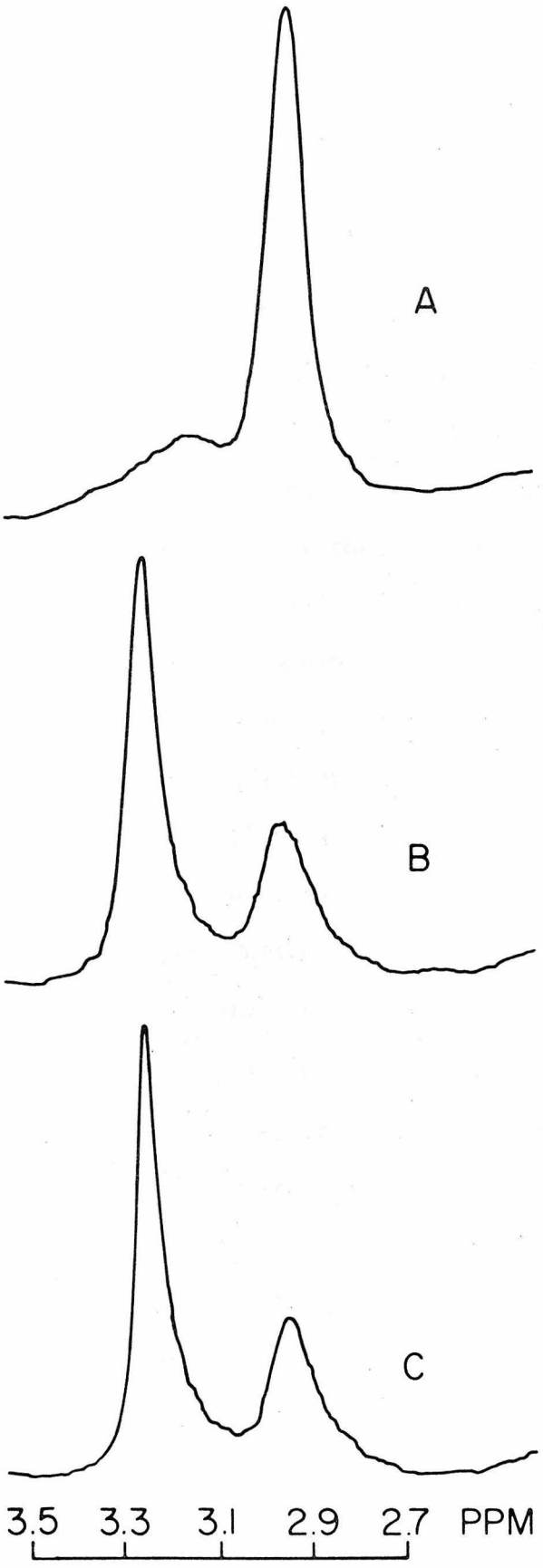
process.

5.5.4 Entrapment of Alamethicin in Small Sonicated Bilayer Vesicles. Alamethicin could be trapped inside small vesicles when the antibiotic-induced fusion is carried out at low antibiotic concentrations ($\sim$ 0.01%) followed by removal of the external alamethicin and ions by dialysis. Under these conditions, only a few fusion steps on the average could have occurred for each of the initial vesicles, so that the sizes of the resultant fused vesicles are still rather small. The concentration of alamethicin trapped within the intravesicular space may be quite high since the surface-to-volume ratio of the enclosed solution remains high. When vesicles are prepared by sonication in the presence of europium (III) ions, followed by subsequent fusion and dialysis of the external medium, a europium (III) ion gradient and an alamethicin gradient are established across the bilayer membrane. These asymmetrical bilayer vesicles can therefore be used to ascertain whether there is transmembrane coupling between the two halves of the bilayer, and whether enough alamethicin pores are present within the bilayer membrane to permit appreciable leakage of entrapped europium (III) ions under these conditions.

The results of such an experiment are summarized in Fig. 17. Because of the trapped europium (III) ions, the pmr spectrum of these

FIGURE 17

100 MHz pmr spectra of the choline methyl protons of lecithin vesicles after fusion by 0.01% alamethicin in the presence of 1.1×10^{-2}M europium (III) ions in both the intra- and extravesicular media (A); and after entrapment of alamethicin and europium ions in the intravesicular solution (B and C). Spectrum (B) was obtained after dialysis of the solution for (A) against NaCl-$La(NO_3)_3$ solution for four hours. Spectrum (C) was obtained after incubation of the solution for (B) at 70° for an additional two hours.


A
B
C
3.5
3.3
3.1
2.9
2.7
PPM

vesicles gives two separate choline resonances with the inner choline methyl proton signal appearing at higher field. The inner choline signal was found to be roughly twice as broad as the outer choline signal. Its integrated intensity was significantly less than expected. Electron microscopy revealed that the average diameter of these vesicles was about 300Å to 350Å, and thus the ratio of the outer vs inner lipid concentration should be about 1.6, but the observed intensity ratio was in fact 1.9. This intensity result, together with the observed broadening of the inner choline resonance, indicates that there is a reduction in the flexibility and mobility of these polar head groups as a result of the alamethicin-lipid interactions. Since no significant broadening or intensity loss of the outer choline signal was observed, we may conclude that any interactions of alamethicin with the bilayer at the inner lipid-aqueous interface are not transmitted to the outer half of the bilayer.

These spectral results confirm our model of alamethicin-induced fusion of vesicles, in which the antibiotics are translocated into the intravesicular solution during fusion. Any interaction of alamethicin with lecithin bilayers is confined principally to the aqueous-bilayer interface. Any appreciable incorporation of the polypeptide within the hydrophobic core of the bilayer would presumably lead to transmembrane coupling between the two halves of the bilayer. Our experiments show that the pmr spectrum of these choline head groups in alamethicin-containing vesicles is essentially unchanged both in chemical shifts and relative intensities even after an incubation period of about two

hours at 70°C. This result indicates that there is no appreciable leakage of the entrapped alamethicin or europium (III) ions into the extravesicular solution, suggesting that there is no extensive alamethicin pore formation under conditions of these experiments.

5.6 Conclusion

In this chapter we have investigated the mechanism of vesicle-vesicle fusion and have demonstrated how an asymmetrical bilayer vesicle can be prepared and be used as a model membrane system. In the next chapter we shall describe an investigation into the pore-forming ability of alamethicin-containing vesicles in the presence of a transmembrane potential difference using these asymmetrical bilayer vesicles.

Chapter VI

VOLTAGE-INDUCED FORMATION OF ALAMETHICIN PORES IN LECITHIN BILAYERS

6.1 Introduction

In the last three chapters we have described various nuclear magnetic resonance studies on the interactions of alamethicin with lecithin bilayer systems. We have shown that the antibiotic is a surface-active agent, interacting with lipid bilayers predominantly in the polar choline region of the bilayer. Such interaction has only minor, if any, perturbation on the molecular packing or motional freedom of the hydrophobic core. This conclusion is substantiated by the results obtained with sonicated bilayer vesicles. In the latter system, which is thermodynamically less stable than the flat multilayers, vesicles readily undergo fusion in the presence of alamethicin which serves as a nucleating agent. In Chapter V we detailed the mechanisms of this fusion process and concluded that some alamethicin molecules actually become translocated from the external solution into the intravesicular space as a result of this alamethicin-induced fusion. Asymmetrical bilayer vesicles can be prepared by this method, followed by extensive dialysis to purge the antibiotic molecules remaining on the outside. In the absence of a transmembrane potential, no evidence was obtained for transmembrane coupling between the two halves of the bilayer or for incorporation of alamethicin into the lipophilic core of the asymmetrical bilayer.

The study of the interaction of alamethicin with phospholipid bilayers would be incomplete without an investigation into the effect of alamethicin on lecithin bilayers when a potential difference is imposed across the bilayer. Since previous electrical studies on alamethicin properties in black lipid films (Gordon and Haydon, 1972; Eisenberg et al, 1973) have established that the ion-conducting channels of alamethicin only function in the presence of a transmembrane potential difference and only if the antibiotic is located on the positive side of the membrane, simulation of this situation in bilayer system would appear necessary in order to correlate the information obtained in the previous chapters with those obtained by electrical measurements.

Sonicated bilayer vesicles offer the advantage of having distinct inner and outer media, and thus a transmembrane potential could be simulated by imposing an ionic gradient across the bilayer. However, the situation here is somewhat complicated by the fact that alamethicin is capable of inducing vesicle fusion if the alamethicin is added externally; any ion-transport via alamethicin pores would be completely obscured by ion-translocation accompanying the predominant process of vesicle fusion. This difficulty may be circumvented, however, if the transmembrane potential is set up on vesicles having alamethicin molecules located inside the enclosed aqueous space only. Preparation of such asymmetrical vesicles is possible, as demonstrated in Chapter V. Bilayer vesicles so prepared are stable. In this chapter we shall report the use of such asymmetrical bilayer vesicles to study the effect of a transmembrane potential difference on the action of alamethicin

on lipid bilayers.

6.2 Sample Preparation

Sonicated DPL bilayer vesicles of average diameter 300Å 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 lecithin per ml D_2O. Sonication with high power at level 4 for about 15 minutes gave a nearly transparent, colorless solution. This was centrifuged for 40 minutes 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° for about 2 hours. The incubated mixture was dialysed 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 three hours. 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 x 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.

6.3 NMR Instrumentation and Electron Microscopy

The Fourier transformed proton magnetic resonance spectra of sonicated lecithin vesicles were obtained with a Varian HR-220 nmr spectrometer operating at 220 MHz for protons, as described in Chapter II. Specimens for electron microscopy were prepared as previously described and were observed on a Philips 201 electron microscope operating at 60 kilovolts.

6.4 Results and Discussion

6.4.1 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 a coupling agent.

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. These asymmetrical vesicles are also stable as long as there exists no major imbalance of ion concentration between the two aqueous media.

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:

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

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 coulomb-mole^{-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.

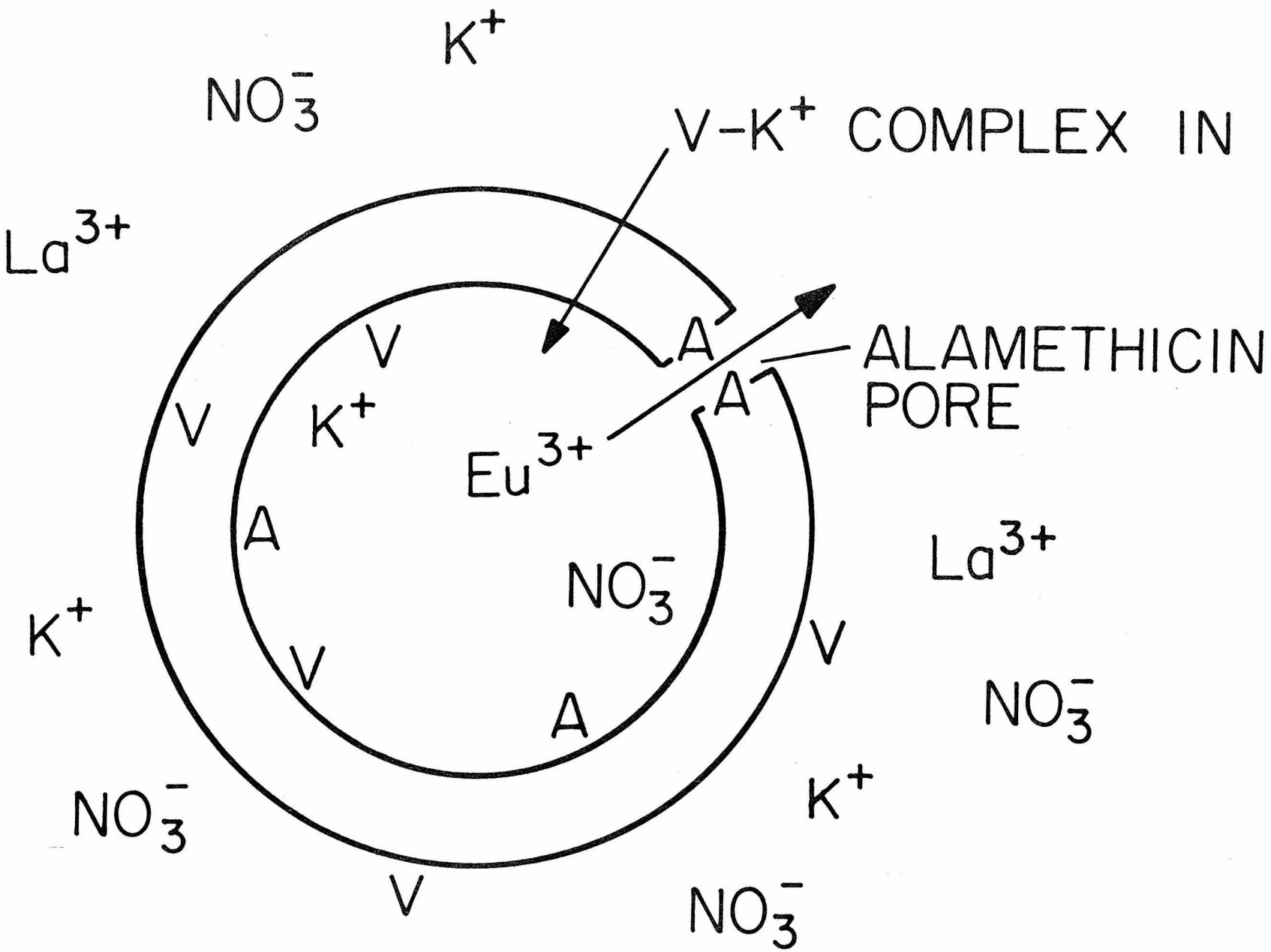
In our experiments the concentration of potassium ions in the outer solution was 0.25 M. Since there were about 6 mM potassium ions in the inner solution (introduced as potassium phosphate buffer), the potential difference across the bilayer is calculated to be about 100 mV. The actual voltage may have 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

FIGURE 18

Schematic representation of the effect of ion-coupler in the creation of a transmembrane potential. Valinomycin transports K^+ inward, establishing a potential difference which triggers the formation of alamethicin pores to allow outward diffusion of Eu^{3+}.

V = valinomycin; A = alamethicin.


K^+
NO_3^-
V-K^+ COMPLEX IN
La^{3+}
V
A
ALAMETHICIN
PORE
A
V
K^+
Eu^{3+}
A
La^{3+}
NO_3^-
K^+
V
V
NO_3^-
A
K^+
NO_3^-
V
NO_3^-

silent flux across lipid bilayers, which would complicate the interpretation of our experimental results.

6.4.2 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 pmr 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 head groups in the other monolayer (Bystrov et al., 1971; Kostelnik and Castellano, 1972; Levine et al., 1973). Thus if europium (III) ions are originally present only in the intravesicular medium 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 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 pmr 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.,

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

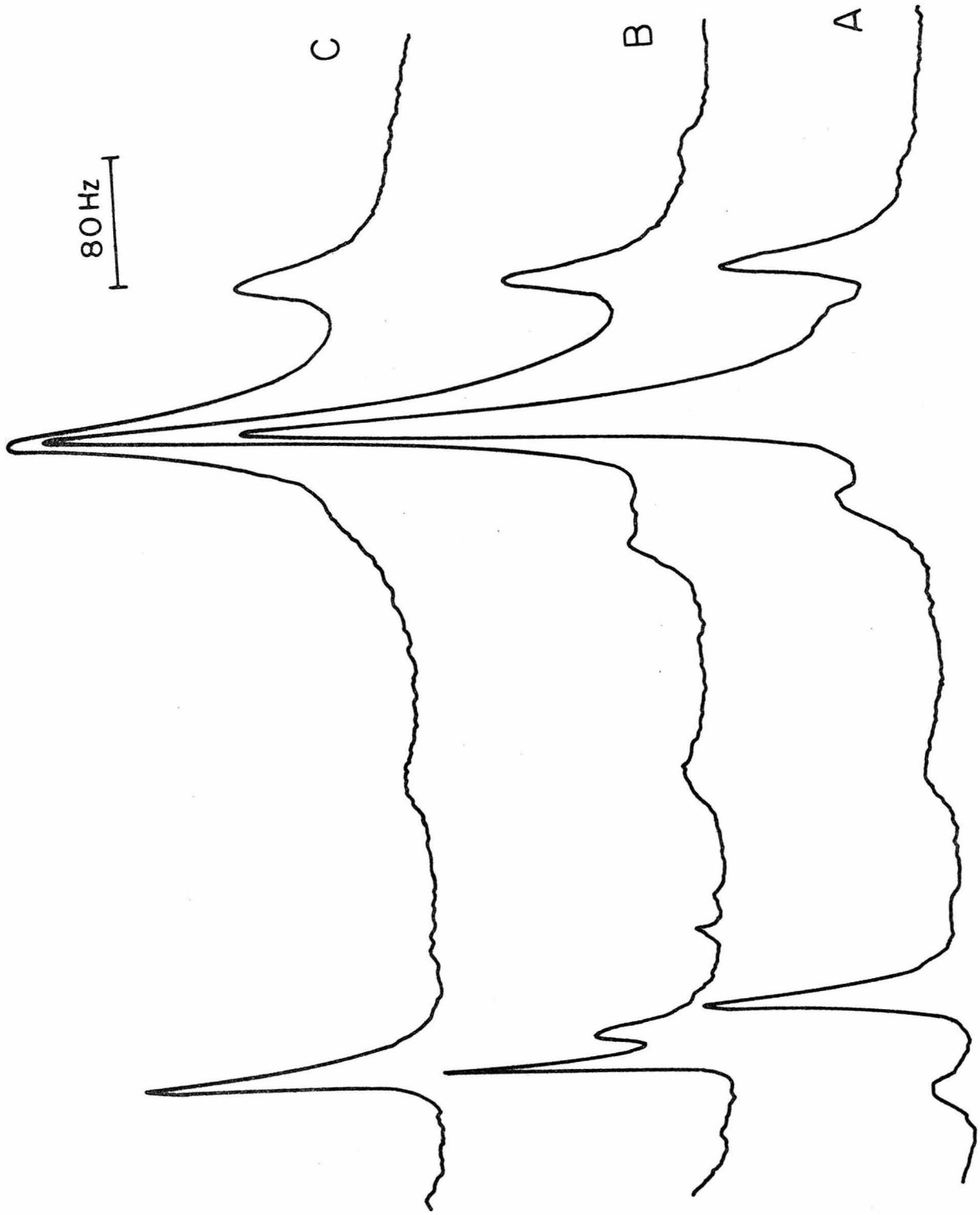
and the extravesicular solution acts as an almost "infinite sink" for the europium ions. Thus, upon the formation of alamethicin pores, europium (III) ions would pass into the extravesicular solution, resulting in a downfield shift of the inner choline proton resonance.

6.4.3 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 19. These data show that when a 0.25 M potassium ion concentration is present outside, europium ions do leak outwards, causing the inner choline pmr 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 pmr signal is not observable by nmr spectroscopy, since at 7 mM rare earth ion concentration each vesicle of average diameter 400Å contains only 600 or so europium

FIGURE 19

PMR 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 two hours. 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 70°.


80 Hz
C
B
A

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.

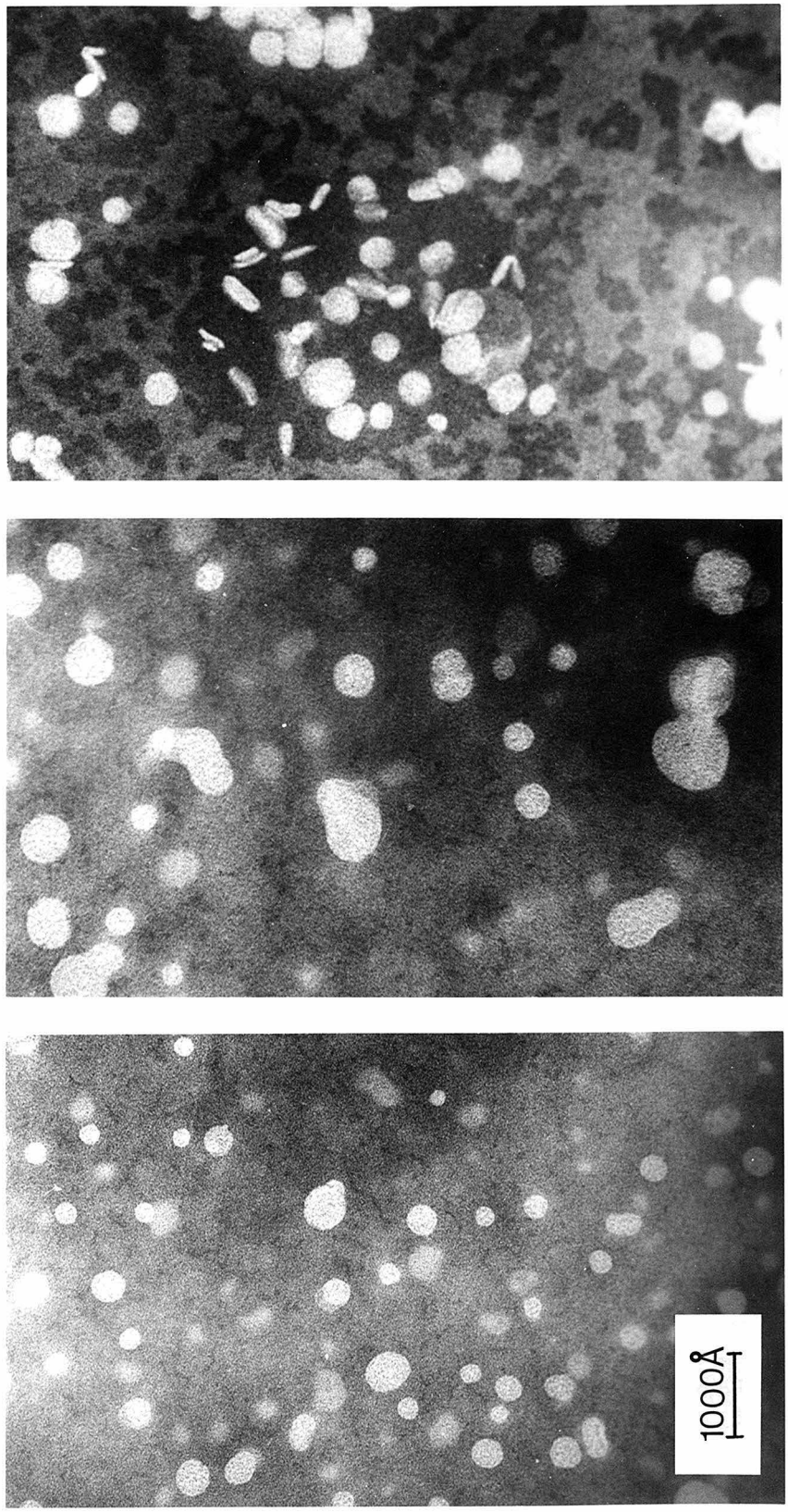
Electron microscopy revealed that some of the smaller vesicles collapsed upon the addition of 0.25 M of potassium nitrate to the exvesicular solution. Figure 20 shows the representative micrographs for sonicated vesicles before (A) and after (B) the alamethicin-induced fusion, and after the establishment of an ionic gradient (C). The rice-shaped particles in Figure 3C represent collapsed vesicles. The fact that these collapsed particles are only observed in Figure 3C indicates that the vesicles are collapsed by the osmotic pressure created by the concentration gradient. Most of the larger vesicles of diameter 400-500Å survived, however. This probably also explains why the fatty acid chain proton signal was somewhat broadened upon the addition of potassium nitrate.

6.4.4 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 pmr 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

FIGURE 20

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 $La(NO_3)_3$. (C) Same sample as in (B) but after 0.25 M KNO_3 added to the external solution.

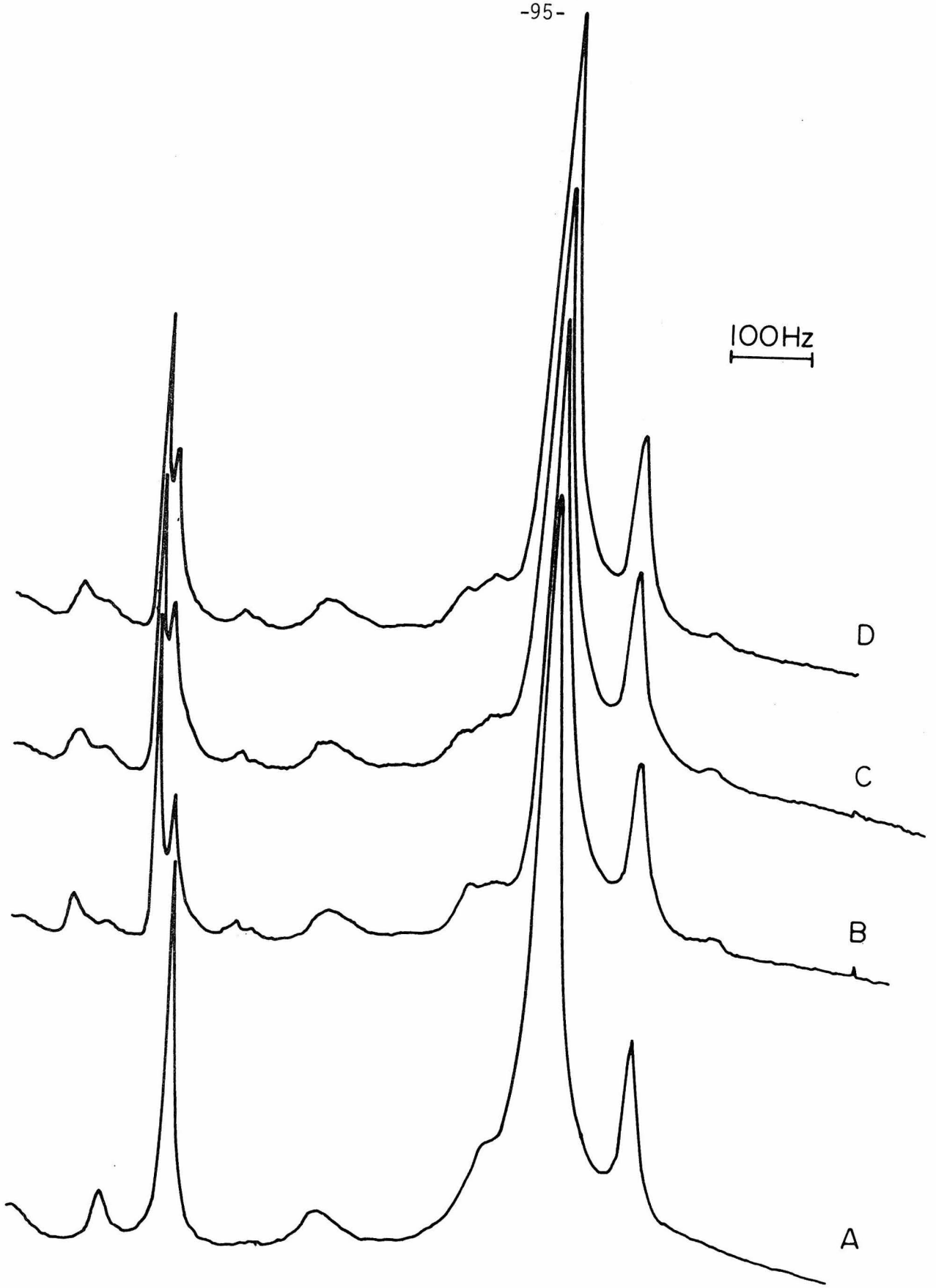

1000Å
A
B
C

over a period of more than half-an-hour after the addition of 0.25 M of potassium nitrate to the extravesicular medium. These results are depicted in Fig. 21. 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 (250-300Å) to give a practically unaltered fatty acid proton signal linewidth 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.

6.4.5 Unidirectionality of the Alamethicin Pore. Once the alamethicin pores are formed upon the application of a transmembrane potential of the appropriate polarity, we can demonstrate that the alamethicin pore is unidirectional, at least for europium ions. This question of the unidirectionality of the alamethicin pore can be ascertained by switching the europium and lanthanum ions between the two compartments in the above experiment. We expect the lanthanum ions to leak outward into the extravesicular solution under these circumstances, but this should have essentially no effect on the spectral features of the choline signals. On the other hand, our method would be particularly sensitive to the inward diffusion of the shift reagent, europium ions, through the alamethicin pores, should this occur, as

FIGURE 21

PMR 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 sample after 5 x 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.


100Hz
D
C
B
A

less than 1000 ions need to be translocated to bring the europium concentration in the inner compartment to the level of that in the outer solution. In this connection we should point out that in this experiment the driving force is very much in favor of the translocation ($\Delta G < 0$) and any unidirectionality of the alamethicin pore must be associated with a high potential barrier toward diffusion across the pore.

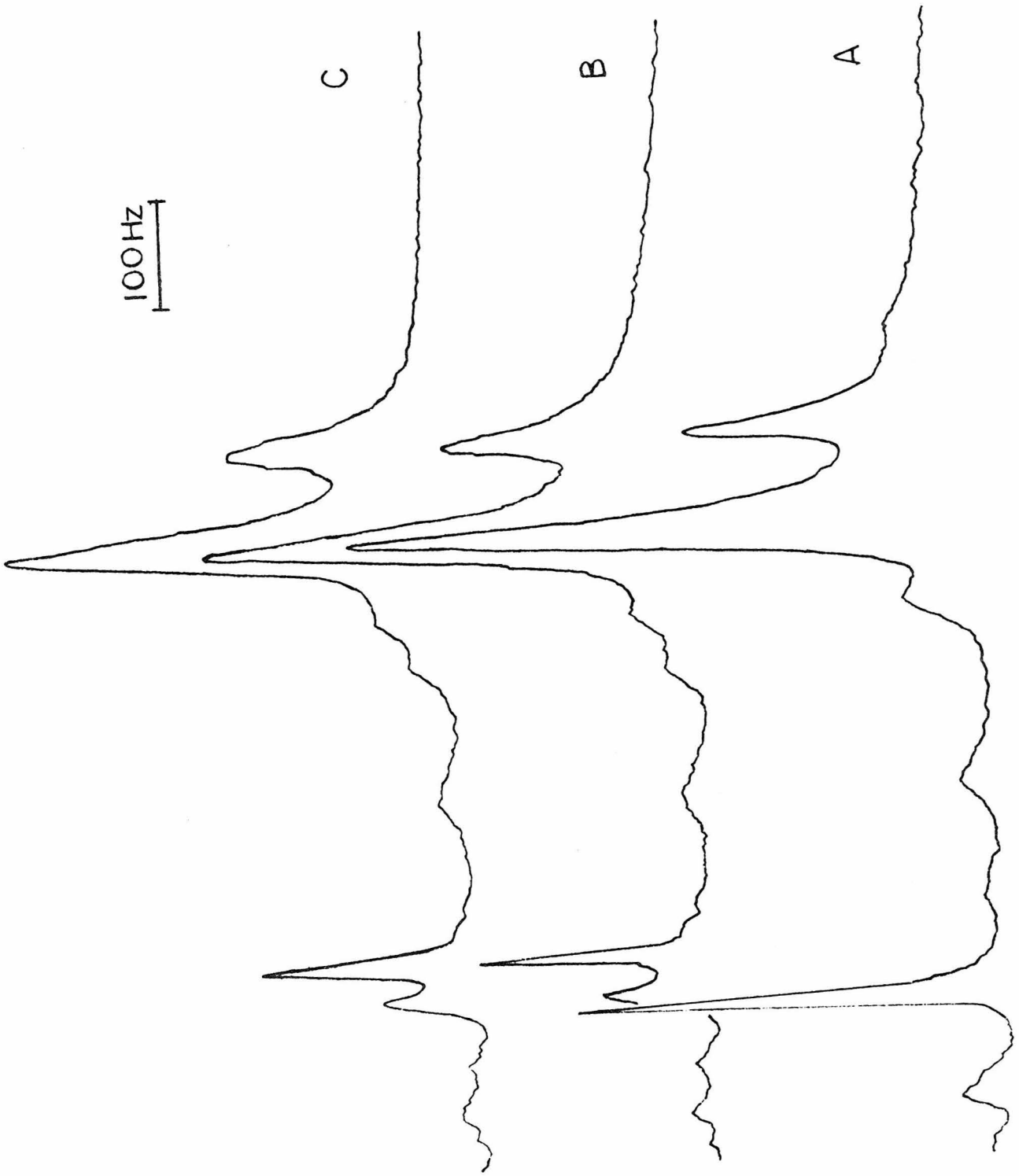
We therefore repeated the experiment by carrying out the sonication and alamethicin-mediated fusion in the presence of lanthanum and the dialysis against europium nitrate solutions. After dialysis we have then europium ions in the external medium and lanthanum ions inside the vesicles. The pmr spectra of these vesicles have the outer choline signal shifted upfield, but the inner choline signal position is unaffected. When a potential difference was imposed by the potassium ion gradient, no significant upfield shift of the inner choline signal was observed. Thus, if the europium is located on the negative side of the bilayer, it cannot pass into the positive region even when an alamethicin pore is open. This result suggests that the highly charged (+3) europium ions encounter a very high potential barrier when they enter the pore in the reverse direction (Fig. 22).

6.5 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

FIGURE 22

PMR spectra (220 MHz) of lecithin bilayer vesicles. (A) Vesicles prepared by sonicating DPL in D_2O with 7 mM $La(NO_3)_3$ and fused with 0.1% alamethicin for 2 hours. (B) Same sample as in (A) but after dialysis against 7 mM $Eu(NO_3)_3$ solution. (C) The dialyzed sample after 5 x 10^{-5}M valinomycin and 0.25 M KNO_3 added to the external solution.


C
B
A
100Hz

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. Alamethicin pores so formed were shown to be unidirectional at least for europium; the cation used to indicate membrane permeability changes in this work.

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 the 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 400Å is on the order of 2×10^4, we do not expect major effects on the observed methylene linewidth, 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 a millisecond need to be formed in each vesicle to account for the results we have observed here.

Chapter VII

PROPERTIES OF ALAMETHICIN IN SOLUTION

We have discussed the interaction of alamethicin with lipid bilayers in the last few chapters. Alamethicin is reversibly adsorbed to the bilayer surface. In a typical black lipid membrane experiment, alamethicin exists in equilibrium among three states: free, adsorbed (pre-pore), and pore. A study of the properties of alamethicin in free aqueous solutions would provide further insight into the subject. Furthermore, alamethicin aggregates extensively in solution; this property has strong implications about its pore-formation mechanism. In this chapter we shall study the pmr spectral characteristics of alamethicin in aqueous media and its aggregation properties.

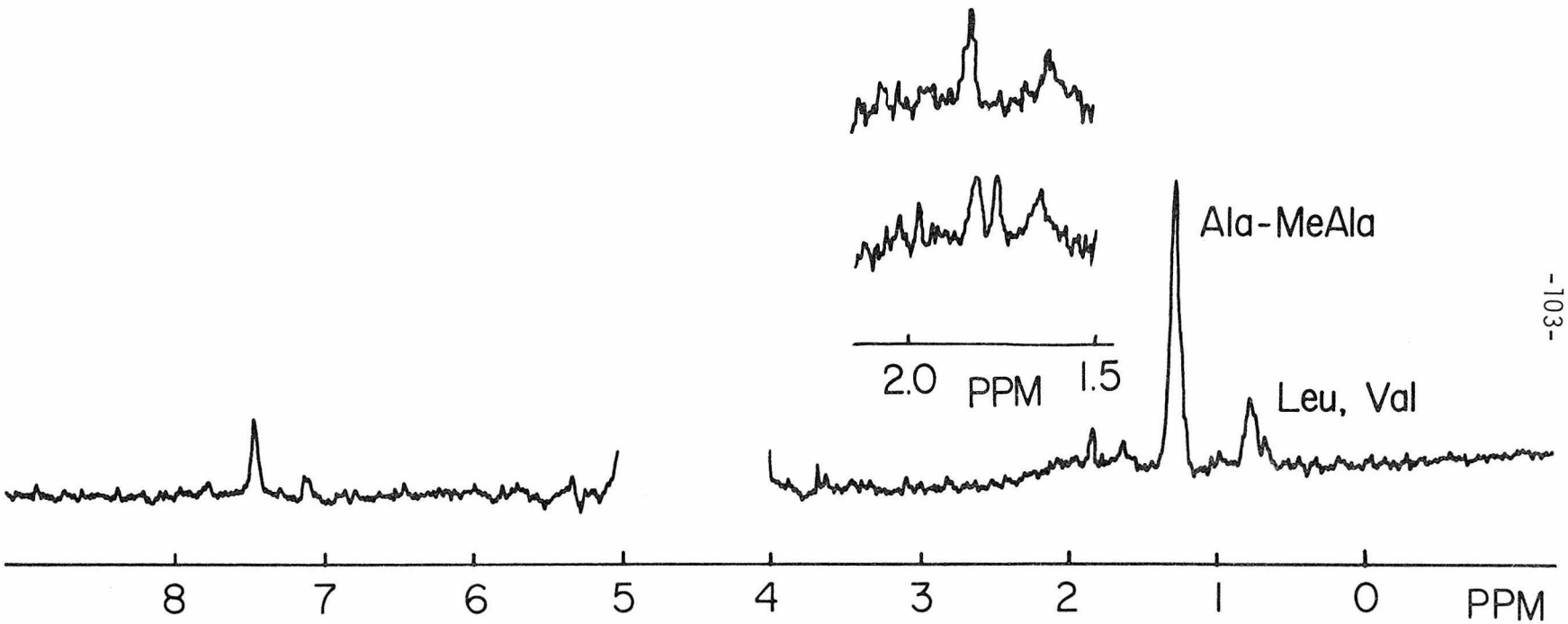
7.1 PMR Spectra of Alamethicin

The high resolution proton magnetic resonance spectrum of alamethicin in D_2O (99.8% ^2H) at 220 MHz is shown in Fig. 23. We have tested, in addition to the two samples from Upjohn Company, a third sample from Microbiological Research Establishment (gift from Dr. G. Christoph, California Institute of Technology). Their Fourier transformed spectra (after 10,000 transients) did reveal minor differences in the spectral region around 1.7 ppm downfield from TMS, probably due to minor variations in the compositions of the polypeptide in the different samples.

The solubility of the antibiotic in aqueous media increases with pH, but pH appears to have no detectable influence on the

FIGURE 23

Fourier transformed pmr spectrum (220 MHz) of alamethicin in D_2O. The immense signal from HOD at 4-5 ppm is omitted. Chemical shifts are measured downfield from an external TMS signal without correction for change in bulk magnetic susceptibility. Region around 1.7 ppm is also shown in expanded scale to illustrate variations in spectral features observed for different batches of alamethicin. Note also the sharp resonance at about 7.4 ppm.


Ala-MeAla
Leu, Val
2.0 PPM 1.5
8
7
6
5
4
3
2
1
0
PPM

polypeptide's spectral characteristics. The pmr spectrum of alamethicin in D_2O containing 10 mM NaOD reveals no significant signal broadening. This contrasts with a somewhat different observation made by Hauser et al. (1970), who reported broadening of the pmr signals under these conditions. We do not know what caused their result, but it cannot have been pH.

We have also observed a rather sharp signal at about 7.4 ppm downfield from TMS, which Hauser et al. (1970) have assigned to the amide protons. However, the fact that this signal persists and remains undiminished even after the sample has been kept in D_2O for over two weeks casts doubt on their assignment. This signal might be due to aromatic residues present in the minor components of alamethicin. Indeed, the recent ^{13}C nmr study by Jung et al. (1975) suggests the existence of phenylalanine or L-phenylalaninol in some components of alamethicin. Their finding was independently confirmed by Martin and Williams (1975). Further studies will be required to resolve this point.

The pmr spectrum of alamethicin in D_2O is essentially unchanged upon addition of potassium or sodium ions, in contrast to the situation with such ionophores as nonactin and valinomycin (Prestegard and Chan, 1969,1970; Haynes et al., 1969). The cations are probably too weakly complexed with the alamethicin to perturb the local magnetic environment of the amino-acid residues significantly; this result agrees with theoretical calculations on alamethicin conformation.

7.2 The Aggregation of Alamethicin in Aqueous Solutions

Alamethicin is reasonably soluble in low dielectric solvents such as alcohols and chloroform. It is less soluble in water, but a concentration of approximately 1 mg/ml can be obtained. The amphipathic nature of alamethicin ensures that it is highly surface-active and aggregates in solutions. Interfacial tension data and ultracentrifugation analysis of alamethicin in aqueous solutions (McMullen and Stirrup, 1971) suggest that some form of aggregation of the antibiotic molecules do indeed take place. In this light, we undertook a study of the effect of ionic strength and pH on this aggregation. The signal due to alanine-methylalanine methyl protons is the most intense in the alamethicin pmr spectrum, and was used by us to determine the amount of non-aggregated polypeptide. We have assumed that the signal intensity is due to the monomers, and that due to higher aggregates are unobservably broad. This alanine-methylalanine signal intensity is measured against a standard hexamethyldisiloxane capillary with varying alamethicin and salt concentrations.

7.2.1 Sample Preparation. An initial alamethicin solution was prepared by dissolving a known quantity of the antibiotic in D_2O with the final volume of solution adjusted to 1 ml. This alamethicin solution was diluted stepwise with neat or saline D_2O as required, and the Fourier transformed spectrum was recorded after each dilution. In experiments where the signal intensity was examined as a function of salt concentration, NaCl was added. The solution pH was modified with NaOD or DCl.

The alamethicin solutions had to be stirred constantly for more than a day to dissolve the polypeptide completely. Increasing the pH expedited solution of the alamethicin.

In the pmr experiments, a capillary containing approximately 10^{-4}M hexamethyldisiloxane (HMDSO) in CCl_4 was used as an external intensity standard. The HMDSO concentration was calibrated against a standard chloroform solution treated with the free radical DPPH.

7.2.2 Results and Discussion. The intensity of the alanine-methylalanine methyl signal of 1-5 x 10^{-5}M alamethicin in aqueous solutions without salt accounts for only about 30-40% (depending on antibiotic concentration) of the value expected on the basis of the stoichiometric amount present. This intensity depended strongly on ionic strength. For example, in aqueous solutions containing 1 M sodium chloride, only 3-6% of the Ala-MeAla signal was observed. We believe that the reduced intensity is due to aggregation of the antibiotic, and we assume that the observed high resolution pmr signal intensities are due to alamethicin monomers alone. Higher aggregates are expected to undergo slower molecular tumbling and hence may not be observable by high resolution nmr.

That the loss in intensity of the Ala-MeAla signal is due to aggregation and not to adsorption of the antibiotic on the glass wall of the nmr tube can be established by filtering the polypeptide solution through finely powdered glass (grain size $\sim$0.1 mm) and then rechecking the intensity of the pmr signal in the filtrate. It was found that five repeated filterings led to no appreciable reduction

of the signal intensity.

Changes in the pH of the solution did not appear to have much effect on the extent of aggregation, even though there was indication of somewhat more extensive aggregation at lower pH's. The inherent limitation in the sensitivity of our method, however, prevented us from making a more conclusive statement on this point.

These pmr results indicated that alamethicin undergoes extensive aggregation in aqueous media. The pronounced intensity loss observed at high ionic strength indicates enhanced aggregation under these conditions. A more direct comparison of our results with those previously reported by McMullen and Stirrup is not feasible, however, since different methods have been used and these methods are sensitive to different ranges of molecular weight; in addition the concentration ranges employed in the two studies are different.

The extent of alamethicin aggregation in aqueous solutions can be estimated on the basis of the observed variation of pmr signal intensity with stoichiometric antibiotic concentration using simplifying assumptions. We invoke stepwise association equilibria of the form

$$A_1 + A_1 \rightleftarrows A_2$$
$$A_2 + A_1 \rightleftarrows A_3$$
$$\vdots$$
$$A_{n-1} + A_1 \rightleftarrows A_n$$

and assume equal equilibrium constants for all steps. Analysis of the pmr data in terms of this model yields stepwise association constants of 6×10^3 and 2×10^5 in the limit of zero ionic strength and at ionic

strength = 1, respectively.

By assuming a stepwise association constant of about 2×10^5 for the aggregation of alamethicin, we calculate that with the stoichiometric alamethicin concentration of 10^{-7}-10^{-6}M (typical concentrations in a black lipid membrane experiment) the decamer concentration varies approximately as the ninth power of the stoichiometric concentration.

7.3 Conclusion

We believe that this finding on the effect of salt on the aggregation of alamethicin has implications on the properties of alamethicin-treated lipid bilayer membranes. It is generally agreed that the so-called alamethicin pores are due to some form of aggregate of the antibiotic. The decrease of characteristic voltage (Eisenberg et al., 1973; Eisenberg, 1973) with increase of alamethicin and/or salt concentration may be explained in terms of an increase in the number of potential pore-forming aggregates as a result of more extensive aggregation of high salt and antibiotic concentrations. We have shown that the decamer concentration varies approximately as the ninth power of stoichiometric concentration. To relate the number of n-mers on the membrane surface n_s to concentration of alamethicin in solution, we invoke a general relationship

$$n_s = \kappa C_a^m C_s^\ell$$

where κ is a constant determined by the type of salt in solution, type of lipid in the membrane, and the membrane surface area; C_a and C_s are the concentrations of alamethicin and salt in the solution. We see

that decamer is the most probable pore-former in a PE-decane membrane used by Eisenberg et al., who found that the number of pores varies with the ninth power of antibiotic concentration. This of course does not rule out aggregates of other sizes as potential pore-formers, but only suggests that the decamer is the most probable. By the same reasoning, the most probable pore-former in a sphingamyelin-tocopherol membrane is either the hexamer or heptamer. In the next chapter we shall present a model of alamethicin pore-formation in lipid bilayer membranes.

Chapter VIII

MODEL FOR ALAMETHICIN PORE FORMATION IN LECITHIN BILAYERS

In Chapter VI we demonstrated that alamethicin forms pores in the presence of a transmembrane potential. Since alamethicin is not incorporated into the hydrophobic interior of the bilayer to any significant extent in the absence of a potential difference, its observed pore-forming ability must be in some way associated with the applied potential. Even though the very small number of alamethicin molecules inside a vesicle renders the usual thermodynamic argument inapplicable in this case, we believe that the experiments in previous chapters provided us with insight into the role of the transmembrane potential in pore-formation. We propose here that the pore is due to aggregates of alamethicin which become imbedded in the bilayer interior as a result of the transmembrane potential. In other words, the electric field is the driving force that pushes the alamethicin aggregate into the bilayer core, disrupting the molecular arrangement of the bilayer and opening up a pore. In this chapter we shall describe a model of alamethicin pore formation in the lipid bilayer system and shall analyze the model in the light of previously reported results from electrical studies of alamethicin-modified black lipid membranes to show that our model is consistent with the results of the other workers.

8.1 Formation of Alamethicin Pores

Our model of pore-formation is based on a few assumptions. We assume that, when alamethicin molecules (and aggregates) in solution

come into contact with the lipid bilayer, most of the molecules and aggregates are adsorbed onto the bilayer-water interface because of the antibiotic's high surface activity:

$$nA(\text{solution}) \rightleftarrows A_n(\text{solution}) \rightleftarrows A_n(\text{adsorbed}) \qquad (1)$$

The adsorption process is reversible with a time constant on the order of minutes. Since the process of pore-formation requires only milliseconds or less, it is safe for all practical purposes to assume that the adsorbed species remains on the bilayer surface. Each alamethicin molecule carries a negative charge on its Glu-17 residue, thus an alamethicin dipole may be formed if it ion-pairs with a positively charged species*. The positive ion is stabilized by the electronegative carbonyl groups of alamethicin. Assuming a 1:1 pairing scheme, the moment μ of this dipole is given by

$$\mu = qd \qquad (2)$$

where q is the effective charge of the dipole, and d is the length of the dipole. Several of these alamethicin-cation complexes may group together to form an aggregate and, when adsorbed, may form a two-

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*Very recently Martin and Williams (1975) presented evidence that alamethicin is in fact a linear polypeptide carrying a negative and a positive charge near either end. With their structure the ion-pairing scheme is unnecessary. Alamethicin would be an electric dipole by itself. Since we have assumed no specific structural detail of the dipole, their results do not invalidate our model. Only in this case is the dipole length longer than in the case of a cyclic alamethicin.

dimensional array on the surface of the bilayer. Either the aggregate forms in solution and is adsorbed en bloc onto the lipid-water interface, or individual adsorbed molecules (and small aggregates) group together by surface diffusion to form a large aggregate.

Up to this point we have assumed no specific orientation of the individual dipoles within the aggregate. Since the free-energy differences between the aggregates on the bilayer surface and those within the hydrocarbon interior is about 20-30 kcal/mole (vide infra), most of the aggregates remain on the bilayer surface.

In the presence of a field, alamethicin aggregates are converted from the pre-pore state to the pore state.

$$A_n(\text{adsorbed, pre-pore}) \rightleftarrows B_n(\text{pore}) \qquad (3)$$

The equilibrium shifts to the right in an electric field. Conformational changes are expected when the aggregate is driven from an aqueous-like environment (in the pre-pore state) into a medium of low dielectric constant (in the pore state) (Jung et al., 1975).

What causes the alamethicin aggregate to transit from the pre-pore state on the bilayer surface to the pore state inside the bilayer core? We believe it is the increased stabilization of an electric dipole in a region of low dielectric constant in the presence of an electric field. We analyze the situation as follows:

In the presence of an electric field E, the potential energy of the dipole is changed by an amount ΔU given by

$$\Delta U = -\vec{\mu} \cdot \vec{E} \qquad (4)$$

We assume that, in the presence of the field, all component dipoles of the alamethicin aggregate become oriented. Each alamethicin molecule assumes the configuration of an elongated loop. If the transmembrane potential is V and the membrane thickness is δ, the electric field within the bilayer region is

$$E = V/\delta \tag{5}$$

since practically all the voltage-drop occurs within the bilayer region. The field in the aqueous region is zero or very nearly so; thus the dipole energy is not lowered in the aqueous medium. Within the bilayer region, however, the drop in potential energy of the dipole aggregate is

$$\Delta U = \mu V/\delta \tag{6}$$

For dipalmitoyllecithin, for example, the bilayer thickness is about 46Å. If the transmembrane potential is 30 mV, the electric field is calculated to be 6.5×10^{4} volt-cm^{-1}. The dipole moment has a magnitude on the order of 5×10^{-25} coul-cm.* Thus the lowering in potential energy of the dipole is

$$\Delta U = -3.3 \times 10^{-20} \text{ joules/aggregate}$$

$$= -4.6 \text{ kcal/mole of aggregate}$$

Similarly if V is 100 mV, ΔU can be calculated to be about -15.5 kcal/mole.

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* An internal check of this value is possible, see below.

This lowering of dipole energy stabilizes alamethicin dipole within the bilayer interior. Thus in the presence of an electric field (with the side on which alamethicin is located being positive) alamethicin is driven into the hydrophobic core of the bilayer, disrupting the bilayer's molecular arrangement to create an entity called a pore (Fig. 24).

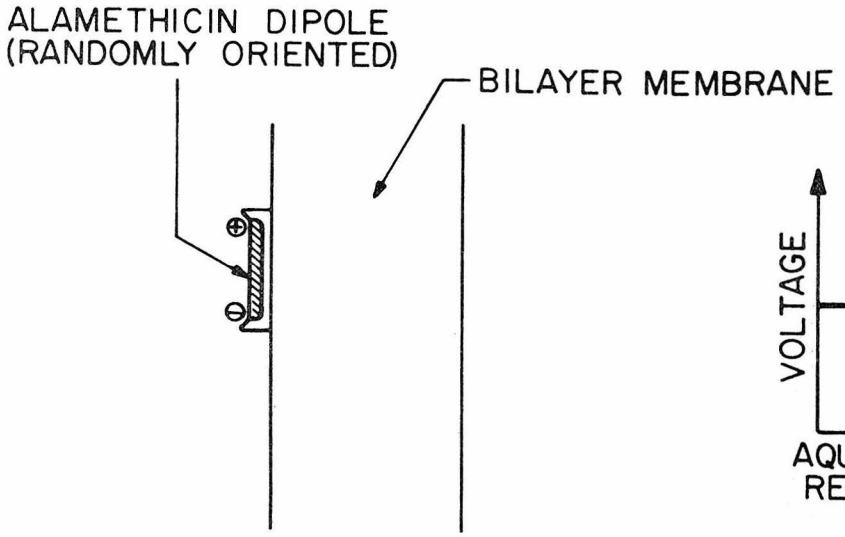
8.2 Structure of the Alamethicin Pore

According to this model, the structure of the pore resembles an open-end barrel, bridging the two aqueous regions through the bilayer, with component molecules lining the "wall" of the barrel. The positive end of each individual dipole is expected to penetrate the membrane interior more deeply because of electrostatic forces.

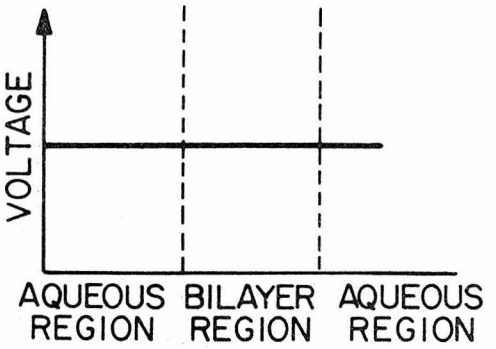
We have assumed that the interior of the alamethicin pores is filled with water. This may be justified by the good agreement of the conductivity and viscosity of the bulk solution and of the contents of the pores. The cross-sectional area of the pore must be large enough for the simultaneous passage of more than one ion (judging from the conductance data and the cation mobilities); hence alamethicin pores have relatively low ion-specificity compared to highly selective ion-carriers such as valinomycin and nonactin. Nevertheless a potential barrier to the passage of ions must exist within the pore. This barrier may be due to one or several sources. The double layer potential near the pore entrance may restrain ions from passing through. The specific interactions of ions with functional groups of alamethicin molecules lining the wall of the pore and a hydrocarbon-like environment within

FIGURE 24

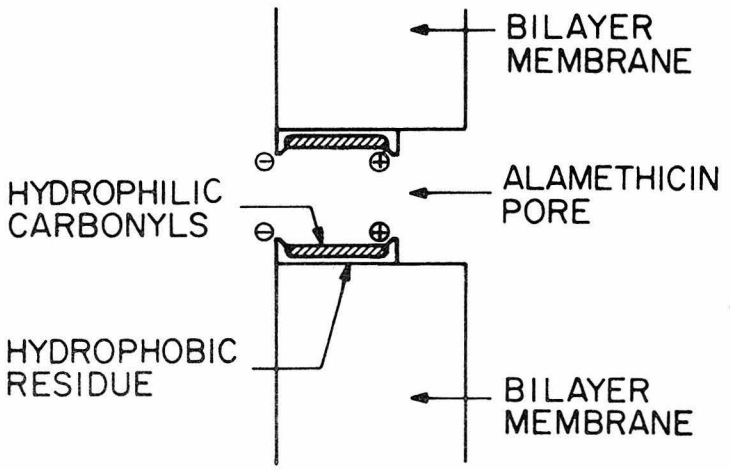
Schematic representation of the action of an electric field on the formation of alamethicin pores in bilayer membranes. In (A) no electric field is present, most of the alamethicin aggregates lie randomly oriented on the bilayer surface. In (B) when a voltage is applied across the bilayer, alamethicin aggregates are driven into the bilayer interior to create a pore. (C) and (D) are the corresponding variations in voltage in (A) and (B) respectively.


ALAMETHICIN DIPOLE
(RANDOMLY ORIENTED)
BILAYER MEMBRANE

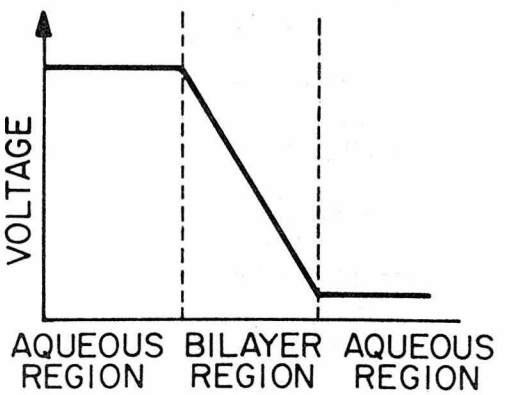
A


VOLTAGE
AQUEOUS REGION
BILAYER REGION
AQUEOUS REGION

C


BILAYER MEMBRANE
HYDROPHILIC CARBONYLS
ALAMETHICIN PORE
HYDROPHOBIC RESIDUE
BILAYER MEMBRANE

B


VOLTAGE
AQUEOUS REGION
BILAYER REGION
AQUEOUS REGION

D

the pore also act as energy barriers to the ions. The hydrocarbon-like environment is a result of the strong electric field which fully orients the solvent molecules inside the pore (Smejtek, 1974). The somewhat different conductance values obtained for the various cations by Eisenberg et al. probably reflect the different degree of influence on the ions by the potential barrier caused by these factors.

Alamethicin aggregates are capable of pore-formation only if the side on which the antibiotic is situated is electrically positive. This reflects the specificity of the response of the dipole to the electric field. We assume that only the positive end of the dipole is driven into the deep interior of the bilayer by an electric field to open up a pore. Electrostatic forces keep the negative end of the dipole near the bilayer surface. The negative end acts as an anchor, preventing the entire aggregate from drifting through the bilayer membrane. This is supported by the fact that alamethicin does not diffuse across the bilayer at any appreciable rate, but the methyl ester of alamethicin does (Lau and Hall, experiments to be published). Moreover, negative charges near the pore entrance lower the potential barrier to cations. Although it is not known how much this contributes to the pore's cation-selectivity, it is expected to be a modifying factor. The methyl ester is less cation-selective, and the corresponding conductivity levels of its pores are lower than those of normal alamethicin pores. Apparently the negative charge on the Glu-17 residue is not vital in pore formation, but it does modify the properties of the pore.

Both Mueller and Rudin (1968) and Eisenberg et al. (1973) reported discrete conductance states of a single alamethicin pore. These are probably due to fluctuations in the cross-sectional area of the pore. A rearrangement of the pore structure resulting in the increase or decrease of the number of alamethicin molecules around the pore would effectively alter the size of the pore's cross-sectional area. We have been discussing this view with Hall and Eisenberg (J. E. Hall and M. Eisenberg, private communications) for some time; this view resembles that of Boheim (1974). Single pore kinetics are beyond the scope of this work, however, We shall be concerned with the mechanism of pore formation in the bilayer only.

8.3 Properties and Predictions of the Model

Eisenberg et al. (1973) have presented a wealth of experimental data on the properties of ion-conducting alamethicin pores. Their study is the most extensive on this subject to date. We shall explain their observations in terms of our model to show the excellent agreement between the two.

Let us define the following parameters:

V_c = characteristic voltage as defined by Eisenberg et al.

n_c = number of pores at the characteristic voltage

n = number of potential pore-formers on the bilayer surface

Λ_c = macroscopic conductance of the membrane at the characteristic voltage

λ = average conductance of a single pore

Since the pore is due to aggregates of alamethicin dipoles driven deep into the interior of the bilayer, we see that

$$n_c = n \exp(-\frac{\Delta G^\circ}{RT}) \exp(-\frac{\Delta U}{RT}) \tag{7}$$

where ΔG° is the molar free-energy difference between the alamethicin aggregates imbedded inside the bilayer core and those adsorbed on the lipid-water interface in the absence of an electric field. (ΔG° is essentially a constant.) ΔU is the change of potential energy per mole of aggregate in the presence of an electric field. Obviously $n_c \leq n$.

If we let Γ denote the quantity $\exp(-\Delta G^\circ/RT)$, we may write

$$n_c = n\Gamma \exp(\frac{\mu E_c}{kT})$$

$$= n\Gamma \exp(\mu V_c/\delta kT) \tag{8}$$

Now $\Lambda_c = n_c\lambda$. Substituting, we have

$$\Lambda_c = n\lambda\, \Gamma \exp(\mu V_c/\delta kT) \tag{9}$$

To relate n to the concentrations of alamethicin (stoichiometric) C_a and of salt C_s in solution, we assume a general expression

$$n = \kappa C_a^m C_s^\ell \tag{10}$$

where κ is a constant which depends on the type of salt in the solution. Hence we have

$$\Lambda_c = \kappa\, C_a^m\, C_s^\ell\, \lambda\, \Gamma \exp(\mu V_c/\delta kT) \tag{11}$$

or

$$\ell n\, \Lambda_c = \ell n(\kappa\, C_a^m\, C_s^\ell\, \lambda\, \Gamma) + (\mu V_c/\delta kT) \tag{12}$$

From equation 12 we can derive an expression relating the characteristic voltage V_c and the stoichiometric concentration of alamethicin C_a :

$$(V_c - V_c') = -\frac{m\delta kT}{\mu}\, \ell n\, \frac{C_a}{C_a'} \tag{13}$$

We see that this equation is identical to

$$V_c = -V_a\, \ell n\, \frac{C_a}{C_o} \qquad \text{(Eq. 1, Eisenberg } \underline{\text{et al.}}\text{, 1973)}$$

with

$$V_a = \frac{m\delta kT}{\mu} \tag{14}$$

and

$$C_o = \left(\frac{\Lambda_c}{\kappa\, C_s^\ell\, \lambda\Gamma}\right)^{1/m} \tag{15}$$

Similarly, an expression relating V_c and the concentration of salt C_s can be derived from eq. 12:

$$(V_c - V_c') = -\frac{\ell\delta kT}{\mu}\, \ell n\, \frac{C_s}{C_s'} \tag{16}$$

Again, this equation is identical to

$$V_c = -V_{salt}\, \ell n\, \frac{C_s}{C_o'} \quad \text{(Eq. 2, Eisenberg } \underline{\text{et al.}}\text{, 1973)}$$

with

$$V_{salt} = \frac{\ell\delta kT}{\mu} \tag{17}$$

and

$$C_o' = \left(\frac{\Lambda_c}{\kappa C_a^m \lambda\Gamma}\right)^{1/\ell} \tag{18}$$

These two expressions (Eqs. 13 and 16) may be combined to yield an equation for the number of pores (n_{pore}) at any voltage V larger than the characteristic voltage. We let $C_a = C_s = 1$, then $\kappa = n$. From this we obtain

$$n_c = \kappa\lambda\Gamma \exp(\mu V_i/\delta kT) \tag{19}$$

where V_i is the characteristic voltage at unit concentrations of alamethicin and salt. Hence the number of pores at any $V \geq V_i$ is given by

$$n_{pore} = n_c C_a^m C_s^\ell \exp\left[\frac{\mu}{\delta kT}(V - V_i)\right] \tag{20}$$

Eisenberg et al. (1973) have determined a ninth power and a fourth power dependency of the number of pores on the concentrations of alamethicin and salt, respectively. Therefore, substituting $m = 9$ and $\ell = 4$, we obtain

$$n_{pore} = n_c C_a^9 C_s^4 \exp\left[\frac{\mu}{\delta kT}(V - V_i)\right] \tag{21}$$

This equation is exactly the same as Equation 24 in Eisenberg et al. (1973), if we make the substitution

$$\frac{\delta kT}{\mu} = V_o = 3.94 \text{ mV} \qquad (22)$$

In equation 22 V_o is a constant independent of the type of salt in the electrolyte.

Eisenberg et al. have obtained numerical values for three constants, V_o, V_a and V_{salt}. We have derived the expressions relating these three constants to the dipole moment μ and membrane thickness δ. We shall use Eisenberg's value for V_o to evaluate μ from which we can determine values for V_a and V_{salt}. We shall see that our model's predictions are consistent with the experimental values.

The expression for V_o is given in equation 25. Assuming a membrane thickness of 46Å, we calculate μ to be

$$\mu = \frac{\delta kT}{3.94} = 4.8 \times 10^{-25} \text{ coul/cm}$$

This value for μ is reasonable for an aggregate consisting of 10 monomers with a dipole length of about 30Å (30Å is the approximate length of an alamethicin molecule from the carboxylic group to the farthest carbonyls in the configuration of an elongated loop).

We have shown that $V_a = m\delta kT/\mu$ (Eq. 14). If we let $m = 9$, we obtain

$$V_a = \frac{m\delta kT}{\mu} = 35.4 \text{ mV}$$

This value of V_a corresponds very well with 36 ± 4 mV, the value experimentally determined by Eisenberg, Hall and Mead (1973).

Similarly, V_{salt} may be calculated from Eq. 17 by putting $\ell = 4$

$$V_{salt} = \frac{\ell\delta kT}{\mu} = 15.8 \text{ mV}$$

Again, we see that this value is well within the error limit of $V_{salt} = 16 \pm 1.5$ mV obtained by Eisenberg et al. (1973).

8.4 Current-Voltage Characteristics According to this Model

Finally, we tested the current-voltage characteristics in our model. We generalize Eq. 11 to get an expression relating Λ, the conductance of an alamethicin-doped membrane, with the applied voltage V:

$$\Lambda = \kappa C_a^m C_s^\ell \lambda\Gamma \exp(\mu V/\delta kT) \tag{23}$$

Since the current, I, is given by $I = \Lambda V$, we multiply both sides by V to get:

$$I = \kappa C_a^m C_s^\ell \lambda\Gamma V \exp(\mu V/\delta kT) \tag{24}$$

This is the expression relating the current through the pores to the applied voltage. We note that κ, C_a^m, C_s^ℓ, λ and Γ are merely a group of parameters which modify the magnitude of the current I at a given voltage V but do not change the shape of the current voltage curves. High concentrations of alamethicin and salt correspond to large values of the product of $\kappa C_a^m C_s^\ell \lambda\Gamma$ and vice versa. Setting $\delta kT/\mu = 3.94$ mV and assuming various values for $\kappa C_a^m C_s^\ell \lambda\Gamma$ we obtained theoretical current-voltage curves shown in Figs. 25 and 26. Small values for $\kappa C_a^m C_s^\ell \lambda\Gamma$ are assumed for the curves in Fig. 25. In this case the current increases exponentially with voltage,

FIGURE 25

Theoretically predicted current-potential curves of alamethicin-treated bilayer membranes. Equation used:

$$I = \kappa\, C_a^m\, C_s^\ell\, \lambda\Gamma\, \exp\left(\frac{\mu V}{\delta kT}\right) V$$

Values of $\kappa C_a^m\, C_s^\ell\, \lambda\Gamma$ assumed for the three curves are: (A) 1×10^{-3}; (B) 1×10^{-2}; (C) 1×10^{-1}.

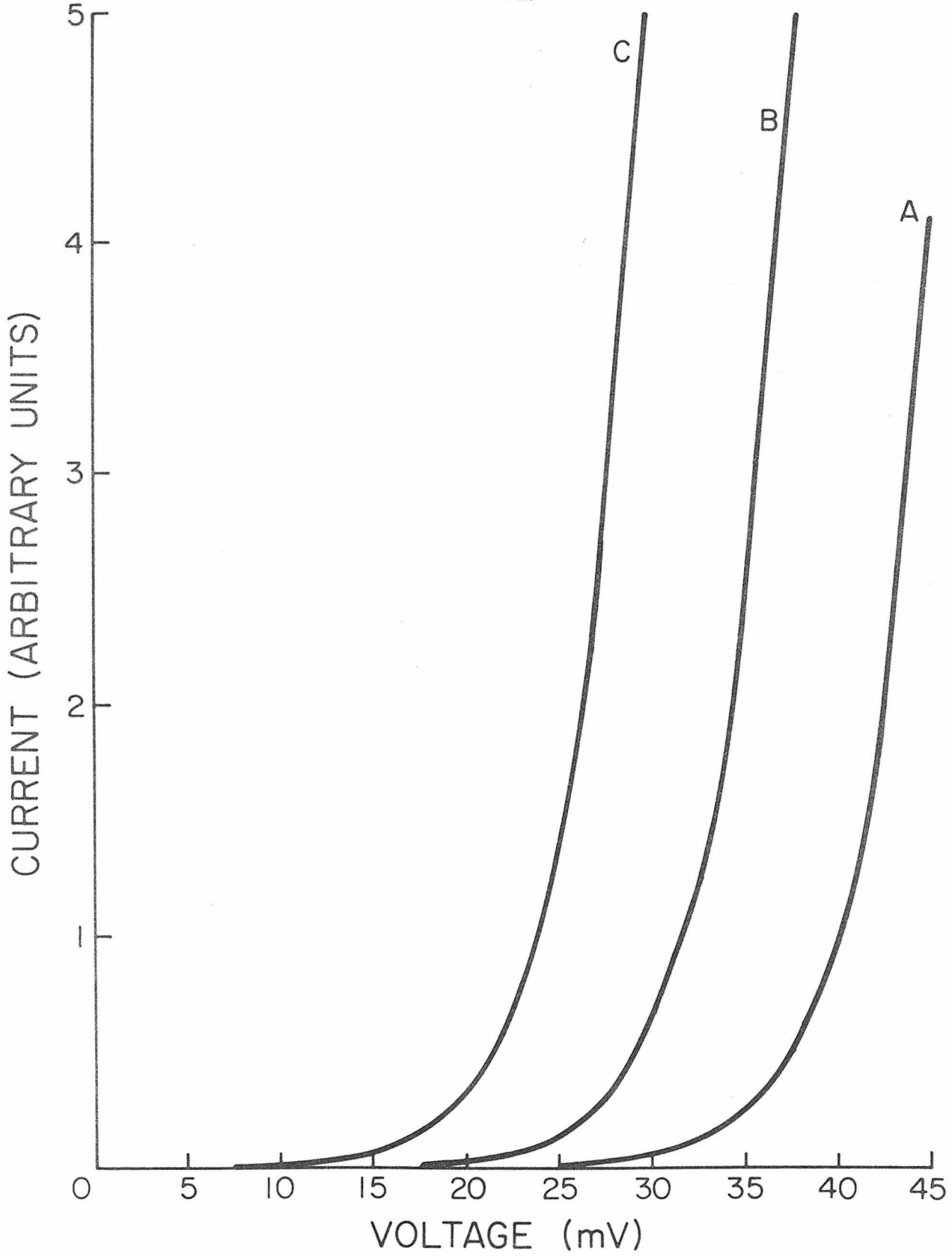
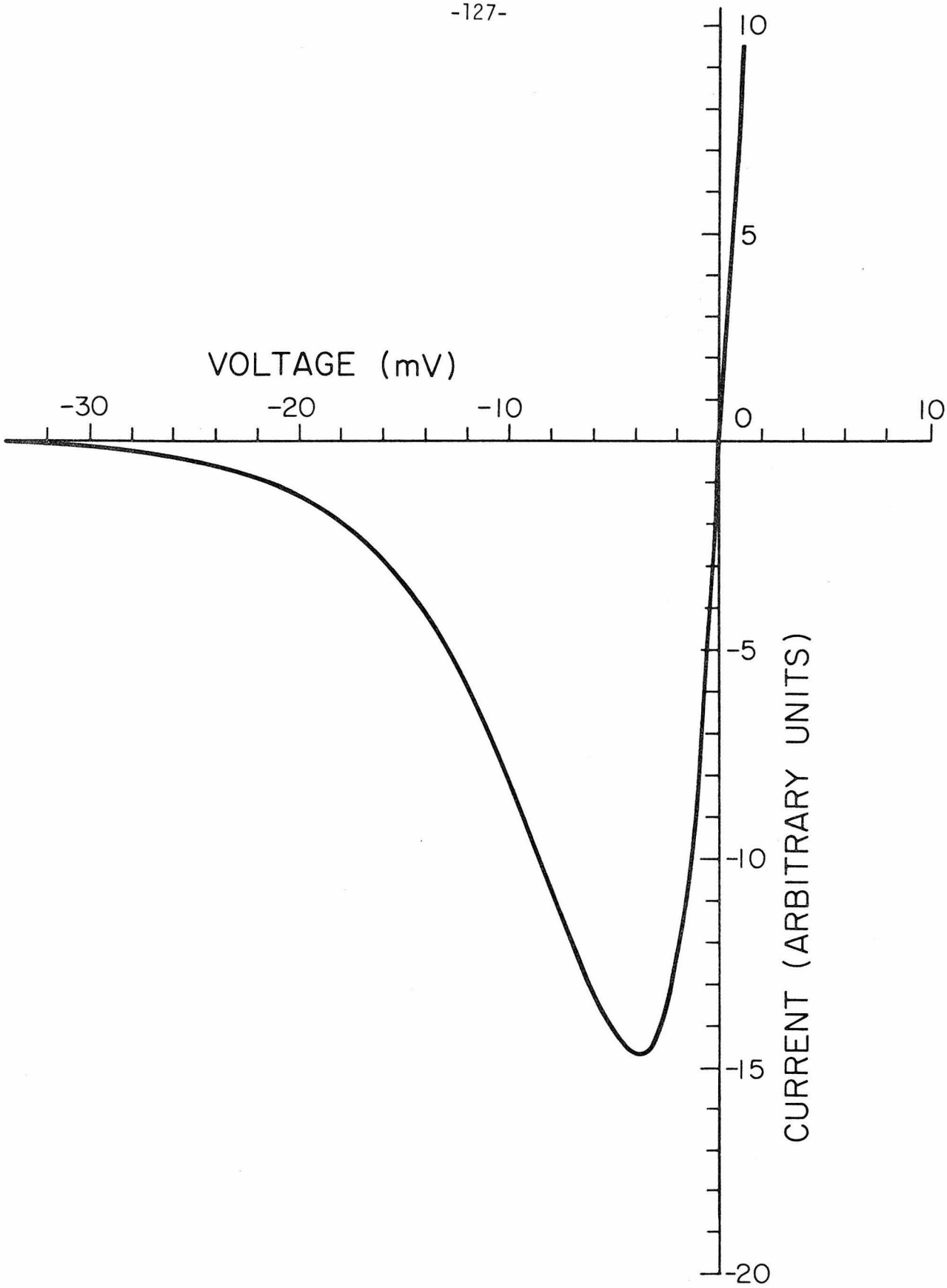

CURRENT (ARBITRARY UNITS)
VOLTAGE (mV)
0
1
2
3
4
5
10
15
20
25
30
35
40
45
A
B
C

FIGURE 26

Theoretically predicted current-potential curve of alamethicin-treated bilayer membranes. Equation used:

$$I = \kappa\, C_a^m\, C_s^\ell\, \lambda\Gamma\, \exp\left(\frac{\mu V}{\delta kT}\right) V$$

Value assumed for $\kappa\, C_a^m\, C_s^\ell\, \lambda\Gamma = 1 \times 10^3$.


10
5
VOLTAGE (mV)
-30
-20
-10
0
10
-5
-10
-15
-20
CURRENT (ARBITRARY UNITS)

resembling the experimental results shown in Fig. 2. If the concentration of alamethicin is large (therefore large $\kappa\, C_a^m\, C_s^\ell\, \lambda\Gamma$), the characteristic voltage becomes negative (Fig. 26), and the current-voltage curve exhibits a region of negative resistance similar to that shown in Fig. 3. In fact, if we let

$$\kappa\, C_a^m\, C_s^\ell\, \lambda\Gamma = 3.03 \times 10^{-7}\text{mho}$$

we find that the curve shown in Fig. 26 agrees quantitatively with that of Fig. 3.

The minimum of the I-V curve in Fig. 26 deserves additional comment. If we differentiate Eq. 24 with respect to V and set $dI/dV = 0$, we see that the current minimum occurs at a voltage V_{min}

$$V_{min} = \delta kT/\mu = 3.94\ \text{mV}$$

This value agrees with that obtained by Eisenberg et al. (1973) (Fig. 3) to within experimental error. This minimum does not show up in Fig. 25 because of the small values for $\kappa\, C_a^m\, C_s^\ell\, \lambda\Gamma$ at low alamethicin concentrations.

From this we may proceed to estimate the value of $\Delta G°$ in Eq. 7. We substitute $C_a = 5.65 \times 10^{-6}$M, $C_s = 1$ M, and let $m = 9$ and $\ell = 4$ (these are the conditions for Fig. 3). We obtain

$$\kappa\lambda\Gamma = 5.17 \times 10^{40}$$

Since λ, the average conductance per pore, has a magnitude on the order of 10^{-9}mho, $\kappa\Gamma = 5.17 \times 10^{49}$. We cannot yet explicitly determine

the value for κ, but it is expected to lie somewhere from 10^{65} (100 pre-pores per 10^{-7}M of alamethicin in solution) to 10^{70} (10^{7} pre-pores per 10^{-7}M alamethicin). Thus ΔG° has a value of 25 ± 5 kcal/mole. This large ΔG° is in fact consistent with the high surface-activity of the antibiotic.

8.5 Conclusion

We have presented and analyzed a model for the voltage-induced formation of alamethicin pores in bilayer membranes. This model successfully accounts for the macroscopic current-voltage behavior of alamethicin-doped membranes. Although ours is based on a dipole model, we have assumed no specific detail of the structure of this dipole. Either a cyclic structure of alamethicin as proposed by Payne et al. (1970) or a linear structure by Martin and Williams (1975) fits into the model equally well. X-ray crystallographic determination of the structure of this polypeptide would be necessary before any structured detail of the dipole may be proposed.

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Proposition 1

FREEZE-FRACTURE ELECTRON MICROSCOPY STUDIES OF LEAKY VESICLES

A new phenomenon in vesicle preparation has recently been observed in this laboratory (Lawaczeck et al., 1975). It was observed that if dipalmitoyllecithin is sonicated at a temperature below that of its phase-transition, and a rare-earth shift reagent such as europium (III) is then added, an anomalous intensity ratio of the two europium-resolved choline pmr signals always results. Negative staining electron microscopy, a technique of established value in visualizing the sizes and shapes of lecithin vesicles (Sheetz and Chan, 1972; Godici and Landsberger, 1974; Lau and Chan, 1974; Papahadjopoulos et al., 1974), yields no meaningful results with these lipid preparations (Hu, V. and Lau, A.L.Y., unpublished experiments). However, if the lecithin is sonicated at a temperature higher than that of its phase-transition, or if the sonicated lecithin preparation is incubated at a temperature much higher than its phase-transition before europium ions are added, the observed choline pmr signals' intensity-ratio correlates well with the average vesicle size (as visualized by electron microscopy).

Further research into this abnormal behavior of vesicle preparations has led to the concept of "structural defects" in lecithin bilayer vesicles. It has been suggested that under abnormal sonicating conditions, lipid molecules group together in different regions of a vesicle, forming "patches". Such patches of lipid bilayers do not match up well at the boundaries, resulting in a phenomenon analogous to phase-separation. This surface inhomogeneity gives rise to cracks along the

vesicular surface; the cracks are large enough for europium ions to pass through. Negative staining electron microscopy does not give meaningful observations presumably because such cracks also allow phosphotungstic acid (the staining reagent) to leak through, destroying the contrast between the vesicle and its surrounding background. Incubation at a high temperature leads to thermal agitation and spatial rearrangement of the lipid molecules, sealing the leaks in the vesicle.

This conclusion is reasonable in view of the ability of some additives to induce phase-separation or related phenomena in bilayer vesicles. Sonication of dipalmitoyllecithin contaminated with more than 1% palmitic acid usually results in vesicles with imperfect structure (viz., abnormal intensity-ratio of the outside to inside choline pmr signals is observed when europium ion is used to resolve them) (Lau, A.L.Y., unpublished experiments).

We propose that freeze-fracture electron microscopy be used to study this phenomenon. The freeze-fracturing technique has been used to observe lipid-lipid interface features of vesicles, such as lipid-protein and cholesterol-lipid interactions (Verkleij et al., 1974). For example, a normal lecithin vesicle has a smooth fractured surface, whereas one containing lipophilic membrane proteins has a fractured surface dotted with protein particles (Vail et al., 1974). A crack in the structure of the vesicle must traverse the entire thickness of the bilayer, and should be observable in freeze-fractured vesicle specimens. Indeed, according to some preliminary results (Hu, V., private communication), fault-lines were observed in some fractured vesicle

surfaces. Although the origin of these lines is still obscure, they may well be due to surface defects. Further research into this problem with freeze-fracture electron microscopy technique could establish this supposition and give additional insight into the structural aspects of the leaky vesicles.

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Proposition 2

ASYMMETRICAL BILAYERS AS A MODEL TO STUDY TRANSMEMBRANE EFFECTS

Since the matrix of a biological membrane is a bilayer of phospholipid molecules, lipid bilayer systems have been used as models for biological membranes. The thermodynamical properties of lipid bilayers have been extensively studied using many physiochemical techniques and such investigations have yielded valuable insight into the structural and functional aspects of membranes (Kornberg and McConnell, 1971; Levine and Wilkins, 1971; Eddidin, 1974; Godici and Landsberger, 1974; Lee et al., 1974; Prestegard and Fellmeth, 1974; Sackmann, 1974; Seelig and Seelig, 1974). The interactions of bilayer systems with various drugs, antibiotics and anesthetics were also investigated and the results of these studies have been fruitful (see, e.g., Hsu and Chan, 1973; Chang and Chan, 1974; Lau and Chan, 1974,1975; Yu et al., 1974; Yu et al., 1975).

Biological membranes have, generally speaking, a bilayer backbone consisting of more than one type of lipid. From studies of bilayer using lipids of different head groups and/or chain lengths it has been established that many properties of membrane-bound proteins and antibiotics are influenced by the types of lipid present. For this reason bilayer systems containing mixed lipids are receiving increasing attention from research workers.

Optical density measurement and differential thermal analysis have shown that lipid vesicles containing mixed lipids have only one thermal phase transition at approximately the weighed average of the various lipids' transition temperatures (Lawaczeck, R., personal communication). That is to say, for example, if dipalmitoyl and dimyristoyl lecithins are well mixed at a 1:1 molar ratio and then sonified, the observed thermal phase transition of the sonified preparation would be around 31-33°, about the average of 42° and 23° which are the phase transition temperatures of these two lipids in their pure state. Hence when lipids are present side by side in a vesicle, their thermodynamic properties are greatly influenced by their neighbors. Rapid lateral diffusion of lipid molecules on the surface of the vesicle apparently can prevent phase separation. On the other hand, if the lipids are sonified separately and then mixed, the mixture exhibits separate thermal transition steps for each lipid present. This indicates that inter-vesicular communication other than vesicle-vesicle fusion is very minimal.

An interesting situation exists when two different lipids are present in the same vesicle, but with each lipid exclusively on only one side of the bilayer. Such asymmetrical bilayer vesicles can indeed be synthesized with the help of lipid exchange proteins, as demonstrated recently by Rothman and Dawidowicz (1975), and have been shown to be stable.

Many interesting experiments can be performed with this asymmetrical vesicle system. For example, it would be worthwhile to determine whether, and to what extent if so, lipids on one side of the bilayer can

modify the properties of lipid molecules on the other side. Since it has been determined that lipid movement traversing the thickness of the bilayer is an extremely slow process, transmembrane communication via a flip-flop mechanism is unlikely (Rothman and Dawidowicz, 1975). If these asymmetrical vesicles display only one thermal phase transition, it would be interesting to find out by what mechanism this can be accomplished. Conversely, if the vesicles show a two-step phase-transition, we would have a situation in which one-half of the bilayer is in a more or less fluid state, while the other half is still in a crystalline phase. This situation, which could arise at temperatures in between the two transitions, is rather difficult to comprehend at this stage. Thus either way the problem is interesting for intellectual thinking.

Other areas of research interest which might utilize these asymmetrical vesicles include the problem of bilayer coupling when the vesicle is acted on by foreign particles. A surface active agent such as alamethicin has been shown not to induce bilayer coupling as long as there exists no major ionic imbalance across the bilayer (Lau and Chan, 1975). Many amphipathic proteins, however, may induce differential response to additives (Sheetz and Singer, 1974). This may be understood from the point of view of bilayer structural disruption caused by these foreign particles. We shall mention one possible experiment as an illustration of how these vesicles may be used in studies of this type.

Valinomycin has been shown to interact quite differently with bilayers of different lipids (Hsu and Chan, 1973). With dipalmitoyllecithin, the action of this antibiotic is essentially confined to the

polar head group region of the bilayer, whereas with dimyristoyllethicin, valinomycin appears to be able to penetrate and break down the bilayer structure. How valinomycin interacts with a vesicle made up of, say dipalmitoyllecithin inside and dimyristoyllecithin outside (or vice versa) would be an intriguing piece of work.

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Proposition 3

ELECTRON MICROSCOPY STUDIES ON VACCINIA VIRUS DNA

The vaccinia virus genome is a double-stranded molecule with a molecular weight on the order of 10^8 (Allision and Burke, 1962; Berns and Rose, 1970; Easterbrook, 1967; McCrea and Lipman, 1967; Sarov and Becker, 1968). After denaturation this DNA appears in the electron micrographs as a full-length circle with a total length twice that of the natural double-stranded species (Geshelin and Berns, 1974). This suggests that either the complementary strands are joined to each other by short segments of single-stranded DNA, or the two complementary strands are covalently linked by some unknown structure at or very near the strands' termini. These linking structures, if they exist, must be strong enough to withstand denaturation cleavage (see Fig. 27a,b).

Vaccinia virus cores are known to synthesize high molecular weight messenger RNA with a long polyriboadenylic acid (poly A) sequence at or near one of its ends. This poly A sequence has an average length of about 200 residues (Kates, 1970; Kates and Beeson, 1970). It is believed that the poly A is synthesized by a transcription from poly dT sites on the vaccinia virus DNA molecule.

We propose to map the sites of the poly dT sequence on this vaccinia virus genome by glyoxal selective blocking technique and electron microscopy. Since poly A is the end sequence of each mRNA, locating the poly dT sites would be equivalent to the mapping of the mRNA coding sites.

The proposed experiments and their reasoning are as follows: It has been known that glyoxal (CHOCHO) can complex with adenosine and

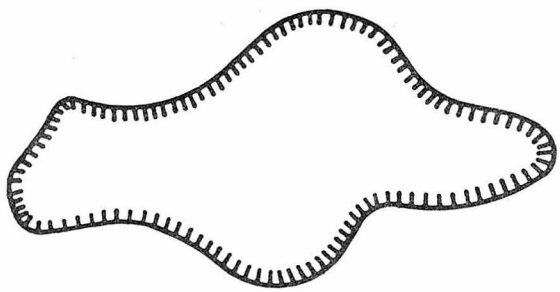
cytidine rapidly and reversibly, but much more slowly with guanosine residues (Hsu et al., 1973). However, the low dissociation constant of the guanosine-glyoxal complex (Broude and Budowsky, 1971) makes glyoxal an ideal blocking agent for G-sites on DNA molecules (Blick and Thomas, 1975). The reason for the irreversibility of the guanosine-glyoxal complexation reaction probably lies in the formation of a stable five-member ring (Hsu et al., 1973). Denaturation of the vaccinia virus DNA by methyl-mercuric hydroxide, a strong base-pair disrupting reagent (Gruenwedel and Davidson, 1966; Kung et al., 1975), is expected to yield a circular single-stranded structure. Reaction of this CH_3HgOH-denatured DNA with glyoxal results in the complexation of glyoxal to essentially all of the dA, dT and dG residues. Only the adduct of guanosine with glyoxal is stable, however. Other glyoxal-nucleotide complexes are readily destroyed by extensive dialysis. Subsequent renaturation of the DNA after dialysis would result in the reassociation of all poly dT sites with their complementary poly dA sequences on the other half of the single-stranded circle. The guanosine residues, however, are irreversibly blocked by glyoxal and thus cannot reassociate with their complementary sequences. Short dA:dT base-pairs would not be stable enough to reassociate, but long poly dA:poly dT pairs would be stable. Thus if such partially renatured DNA molecules are spread from an appropriate formamide-Tris solution and observed under an electron microscope, each molecule would appear as a many-looped chain. The reassociated poly dT:poly dA base-pairs would be observed as the nodes in the chain (Fig. 27d schematically represents these reassociated sequences as short double-stranded threads but in reality, because of the resolution of the

electron microscope involved, they would appear merely as nodes in the chain.)

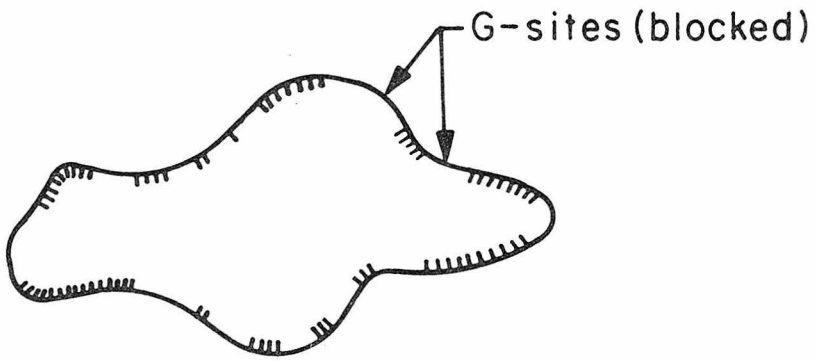
FIGURE 27

Schematic representation of the structure of vaccinia virus DNA under different conditions: (a) In the natural form, the DNA is double-stranded with complementary strands interconnected. (b) In the denatured form it appears circular. (c) After treatment with glyoxal, G-residues are permanently blocked. (d) Renaturation results in the formation of a many-looped chain.

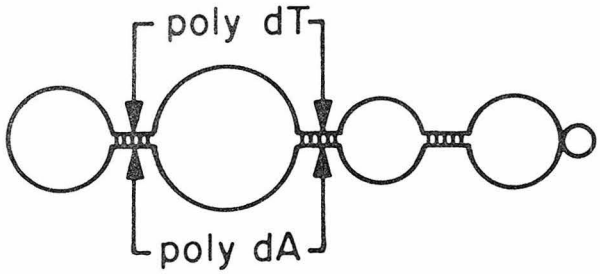
(a)



(b)



(c)



(d)

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Proposition 4

SYNTHESIS OF SPECIFICALLY LABELED LIPID MOLECULES

Lipid bilayer systems have been receiving much attention in biophysical research in recent years. It is thought that lipid bilayers represent the structural skeleton of biological membranes (Levine and Wilkins, 1971; Kuhn and Mobius, 1971). For this reason much effort was made to investigate the thermodynamic properties of bilayer systems, especially the state of motion of component molecules of the bilayer membrane under various experimental conditions. Nuclear magnetic resonance spectroscopy, by virtue of its sensitivity to structural details and environmental changes, has been one of the most widely used techniques in studying lipid bilayer systems (see, for example, Chan et al., 1971, 1973; Henderson et al., 1974; Horwitz et al., 1972; Lee et al., 1972; Lichtenberg et al., 1975).

In nmr studies of lipid bilayers, the hydrocarbon chain region has usually been treated as a single unit, despite the fact that this region occupies some 70% or more of the entire bilayer thickness. Because of the motional sluggishness of the chain protons and the magnetic anisotropy within the region (Kaufmann et al., 1970) it has not been possible to resolve the broad hydrocarbon chain nmr signal into various components. For example, existing data on multilayer systems show only a single longitudinal relaxation time for all methylene protons, even though it is generally accepted that protons at different chain positions should experience different magnetic environment and thus should have different relaxation times.

We propose that specifically ^{13}C-labeled lipids be synthesized and utilized to study the nature of the hydrophobic interior of the bilayers and its interaction with intrinsic membrane proteins, ionophores, etc. ^{13}C-labeled lipid bilayer is advantageous in having the region under investigation well defined. This labeling technique is superior to other methods in that the bilayer structure remains essentially unaltered. Other labeling methods such as ^{19}F substitution for protons (Gent et al., 1975), or addition of covalently linked nitrosyl radicals (i.e., electron spin resonance method) are with the inherent uncertainty of having the bilayer structure disturbed by these additives or substitutions. Even perdeuterated lipids have substantially different phase-transition temperatures (Petersen et al., 1975).

The main difficulty in the synthesis of labeled lipids is in the synthesis of the corresponding fatty acids. The addition of the fatty acid to the glycerophosphorylcholine to form the lipid is relatively straightforward. We propose the following routes of synthesis:

(A) Synthesis of fatty acids with the ^{13}C-label near the carboxyl group:

$$CH_3(CH_2)_nOH + 48\%\ HBr \longrightarrow CH_3(CH_2)_nBr + H_2O \qquad (1)$$

$$CH_3(CH_2)_nBr + Mg/Ether \longrightarrow CH_3(CH_2)_nMgBr \qquad (2)$$

$$CH_3(CH_2)_nMgBr + {}^*CH_2O \longrightarrow CH_3(CH_2)_n\ {}^*CH_2OH \qquad (3)$$

$CH_3(CH_2)_n{}^*CH_2OH$ can undergo the Grignard synthesis once more to give $CH_3(CH_2)_n{}^*CH_2CH_2OH$ which on oxidation by chromic acid gives the fatty acid $CH_3(CH_2)_n{}^*CH_2COOH$.

Alternate route from step 2:

$$CH_3(CH_2)_nMgBr + *CO_2 \longrightarrow CH_3(CH_2)_n*COOH \qquad (4)$$

which puts the labeled carbon on the carboxylic group.

(B) Synthesis of fatty acids with the ^{13}C-label near the terminal methyl group:

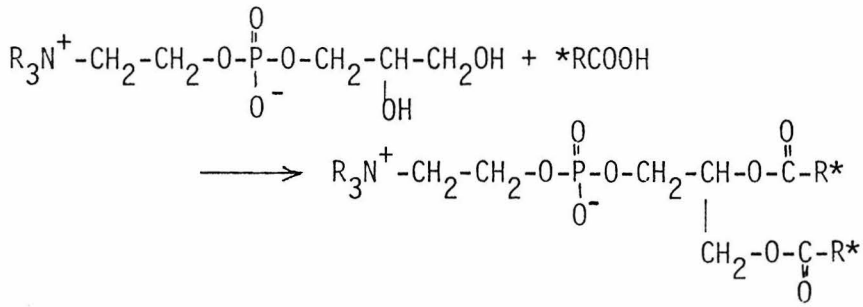
$$RCH{=}CH(CH_2)_nBr + Mg/Ether \longrightarrow RCH{=}CH(CH_2)_nMgBr \qquad (5)$$

$$RCH{=}CH(CH_2)_nMgBr + C_6H_5SO_3*CH_3 \longrightarrow RCH{=}CH(CH_2)_n*CH_3 + C_6H_5SO_3MgBr \qquad (6)$$

Ozonization will break the double bond to give the aldehyde $*CH_3(CH_2)_nCHO$ which is readily oxidized to give the desired product, $*CH_3(CH_2)_nCOOH$.

(C) Synthesis of the ^{13}C-labeled lipid:

After the labeled fatty acid is synthesized, the corresponding monoacid lecithin can readily be prepared by reacylation of sn-glycero-3-phosphorylcholine (GPC):



$$\longrightarrow R_3N^+-CH_2-CH_2-O-\overset{\overset{O}{\|}}{\underset{\underset{O^-}{|}}{P}}-O-CH_2-\underset{\underset{CH_2-O-\overset{\overset{O}{\|}}{C}-R*}{|}}{CH}-O-\overset{\overset{O}{\|}}{C}-R* \qquad (7)$$

GPC is obtained from deacylation of purified egg-lecithin (Kroon, 1975).

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Proposition 5

CRYSTAL STRUCTURES OF A23187-METAL COMPLEXES

The ionophore A23187 has been shown to transport divalent cations across biological membranes (Reed, 1972; Caswell and Pressman, 1972; Scarpa et al., 1972; Wong et al., 1973). It can also uncouple oxidative phosphorylation and inhibit ATPase in rate liver mitochondria (Reed and Lardy, 1972; Wong et al, 1973). The molecular composition of this ionophore has been determined to be $C_{29}H_{36}N_3O_6^-$ (Chaney et al., 1974). Titration and fluorescence studies have indicated that the ionophore complexes with divalent cations in a 2:1 ratio in nonpolar solvents (Puskin and Gunter, 1975).

Ionophore A23187 is unique in the aspect that it is specific for divalent cations. The complexation reactions of it with transition metal ions such as Cu^{2+} and Mn^{2+} have been studied by electron spin resonance spectroscopy (Puskin and Gunter, 1975). It is shown that the coordination between the metal ion and the ionophore is predominantly ionic in character. Since the anionic form of A23187 carries a negative charge, it is believed that it is this neutral 2:1 (ionophore/cation) complex that is responsible for the transit of divalent cations across the lipid membrane phase. The crystal structure of this antibiotic has not yet been determined, but indirect methods such as mass spectroscopy and nmr spectroscopy indicated that its structure is probably similar to that of the polyethers.

It is proposed that the crystal structure of this ionophore in its complexed form in solvents of different dielectric constants be deter-

mined. Membrane-bound antibiotics are expected to exhibit different conformational characteristics in aqueous solutions and in solutions of lower dielectric constants and higher viscosities. For example, alamethicin (another antibiotic which is a pore-former) was shown to exist in different forms in water as compared to in more hydrocarbon-like solvents (Jung et al., 1975). For ion carriers, such as valinomycin and nonactin, the complexed forms of these antibiotics are known to give different nmr spectral characteristics than their free forms (Haynes et al., 1969; Prestegard and Chan, 1969,1970). Conformational changes in different solvents is especially possible in the case of A23187 since it has been suggested that the transport process of this ionophore involves first the formation of a 1:1 complex in the polar aqueous region followed by binding of a second A23187 molecule near the polar bilayer surface to form a 2:1 complex. The neutral 2:1 complex is then extracted into the nonpolar membrane interior (Puskin and Gunter, 1975). It is expected that different conformations of this ion-carrier exist in different solvents. In aqueous solutions, where a 1:1 complex is more probable, the structure of this ionophore is expected to be different from that in a more hydrocarbon-like solvent such as chloroform or 1-octanol. Investigation on the structural aspect of A23187-divalent cation complexes in various solvents should provide us with insight in the ion-transport properties of this antibiotic.

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