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Nuclear Magnetic Resonance Studies of the Interaction of Alamethicin with Lecithin Bilayers†

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ABSTRACT: The interaction of alamethicin with both unsonicated lecithin multilayers and sonicated bilayer vesicles has been investigated by nuclear magnetic resonance (nmr) spectroscopy and electron microscopy. It is shown that alamethicin is a surface active agent, which interacts primarily with the polar choline head groups of the lecithins. Alamethicin also induces aggregation and subsequent fusion of small bilayer vesicles ~300 Å in diameter, a process which was found to have a profound influence on the nmr spectral properties of these bilayers.

Alamethicin (Payne *et al.*, 1970), an extracellular macrocyclic polypeptide from the fungus *Trichoderma viride* (Meyer and Reusser, 1967), has been shown to induce ion movements across certain biological membranes (Pressman, 1968). It has also been shown that this antibiotic interacts with black lipid membranes to form voltage gateable ion channels with discrete conductance states (Muller and Rudin, 1968; Gordon and Haydon, 1972). Since alamethicin itself is not known to be translocated across a black lipid membrane, it has been proposed that voltage gateable ion pores are induced by alamethicin (Eisenberg *et al.*, 1973).

A knowledge of the mode of interaction of alamethicin with phospholipid bilayer membranes is essential toward the understanding of the action of this antibiotic. Various methods may be used to elucidate this interaction, including electrical measurements (Eisenberg *et al.*, 1973; Lau and Hall, 1974), X-ray diffraction and differential scanning calorimetry (Chapman *et al.*, 1969), electron spin resonance (esr) spin labeling (Finer *et al.*, 1969; Levine *et al.*, 1973), circular dichroism (McMullen *et al.*, 1971), and fluorescence techniques (Case *et al.*, 1974). Nuclear magnetic resonance (nmr) spectroscopy, however, offers unique advantages by virtue of its sensitivity toward unravelling structural details and environmental changes. This sensitivity, for example, has been exploited in many of the nmr investigations of the structural and dynamic properties of lecithin bilayers (Penkett *et al.*, 1968; Chan *et al.*, 1971; Lee *et al.*, 1972, 1973; Levine *et al.*, 1972; Horwitz *et al.*, 1972; Seiter and Chan, 1973; Michaelson *et al.*, 1973; Feigenson and Chan, 1974; Seelig and Seelig, 1974). More recently, proton magnetic resonance (pmr) spectroscopy has also been used to monitor the interaction of various oligopeptides with lipid multilayers and phospholipid bilayers (Hsu and Chan, 1973; Chang and Chan, 1974). This present work is concerned with a similar pmr study of the interaction of alamethicin with dipalmitoyllecithin bilayers in both the sonicated and unsonicated states.

Unsonicated liposomes and sonicated bilayer vesicles offer two different, although interrelated, model membrane systems. In terms of bilayer structure these two model membrane systems differ primarily in their surface curvatures (Sheetz and Chan, 1972). It has been pointed out that 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). More specifically, it has been argued that the packing arrangements of these molecules in flat bilayers are more regular than in bilayer vesicles ~300 Å 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 chains is relatively restricted in the case of flat bilayers, this motion is much freer in small bilayer vesicles and the motional state of the hydrocarbon region in the sonicated vesicles is thought to very nearly resemble that of a

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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 would seem to be in order.

By virtue of their high surface curvature, small bilayer vesicles are expected to be intrinsically less stable thermodynamically than their unsonicated lamellar counterpart. In fact, evidence is accumulating to indicate that small bilayer vesicles do have the tendency to undergo vesicle–vesicle fusion (Prestegard and Fellmeth, 1974), although the rate of this fusion process can be controlled somewhat by appropriate choice 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 can become more probable. Thus, any study of the interaction of alamethicin with sonicated bilayer vesicles such as that previously reported by Hauser and coworkers (1970) would be incomplete without a careful examination of this possibility of alamethicin-promoted vesicle fusion. This point will be considered in some detail using electron microscopy (EM) in this present work.

Experimental Section

Materials. Alamethicin was obtained as a gift from Dr. G. B. Whitfield of The Upjohn Company (sample No. U-22324.8831-CEM-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–$CHCl_3$ in a ratio of 1:9, and finally by 1500 ml of CH_3OH–$CHCl_3$ (9:1). The lecithin was found in the last eluent. After rotary evaporation of the eluting solvent a white product was obtained and its purity was checked by thin-layer chromatography.

D_2O (100%) was purchased from Stohler Isotope Chemicals. CH_3OH and $CHCl_3$ were Matheson Coleman and Bell spectrograde products. NaCl was Baker analyzed reagent grade product. All were used as received.

Sample Preparation. (i) ALAMETHICIN IN UNSONICATED LIPID BILAYERS. A known amount of the purified lecithin was weighed into an nmr tube attached with a 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 well mixed by vortexing and let stand overnight to achieve equilibrium. After capillaries containing $CHCl_3$ doped in DPPH (area standard), Silanor C (chemical-shift standard), and ethylene glycol (temperature standard) were added to the sample, the lecithin–chloroform solution was vacuum pumped for 48 hr to remove the solvent. Heating the sample to 50–60° during evacuation was found to be necessary to get rid of the last trace of chloroform and methanol used in the chromatography purification. D_2O (100%) was then transferred into the sample to obtain an ~25% (w/v) lecithin suspension. After the tube was sealed under nitrogen at a pressure slightly lower than atmosphere, it was heated in a water bath to about 60°, and the suspension was homogenized by vortexing.

(ii) ALAMETHICIN WITH SONICATED BILAYER VESICLES. Sonicated bilayer vesicles were prepared using a Branson sonifier equipped with microtip. A known quantity of purified lecithin was weighed into a centrifuge tube, to which D_2O containing ~2 mM phosphate (sodium salt) at pD 7.4 was added to give a suspension of about 50 mg in lecithin/ml of D_2O. Sonication at level 4 for about 15 min produced a nearly transparent, colorless solution. This was centrifuged at 12,000 rpm in a Superspeed RC-2 centrifuge for 40 min 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 finally added to a sonicated preparation of lecithin vesicles. This resultant mixture was then equilibrated using a vortex mixer for less than 1 min 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 vesicle solutions were performed using the dry weight method. After the addition of alamethicin, lecithin and alamethicin concentrations 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 of the order of a few hundredths of a milliliter *vs.* a total volume of 4 ml and the alamethicin concentration used was low (~10^{-4} M).

Pmr Spectra. The delayed Fourier transform (DFT) spectra of unsonicated lecithin bilayers were obtained with a Varian HR-220 nmr spectrometer equipped with Fourier transform accessories and interfaced to a Varian 620i computer. A data collection delay time of 346 μsec was introduced in the DFT experiments in order to filter out the broad methylene component in the pmr spectrum. The use of the DFT method to permit observation of the sharper methyl signals from unsonicated lecithin bilayer systems has been adequately documented elsewhere (Chan *et al.*, 1971, 1973; Hsu and Chan, 1973). In these experiments the intensities of both the choline methyl and terminal methyl proton resonances were measured against external chloroform–DPPH intensity standard, and were corrected for the receiver deadtime introduced into the spectrometer.

Spin–spin and spin–lattice relaxation times were measured using a 54-MHz pulse nmr spectrometer purchased from Tomlinson Research Instrument Co.

Pmr spectra of sonicated lipid vesicles were recorded on the Varian HR-220 nmr spectrometer operating in the continuous wave mode. The intensities of these signals were determined against a chloroform–DPPH external reference. All spectra were recorded within 30 min after the mixing of alamethicin and vesicle solutions.

In all of the above experiments, sample temperature was controlled with a Varian 4540 variable temperature controller.

Electron microscopy has been used to ascertain the size distribution and the state of aggregation of bilayer vesicles.

Immediately before and after each nmr experiment, a portion of the vesicle solution was diluted to 1–5 mg/ml in lecithin. 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 observable under the electron microscope. A drop of this resultant

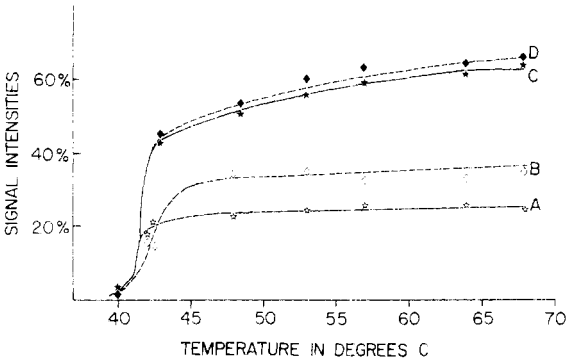

FIGURE 1: Variations of the intensities of choline and terminal methyl proton signals of unsonicated dipalmitoyllecithin bilayers with temperature: (A) choline methyl protons without alamethicin; (B) choline methyl protons in the presence of 1% alamethicin; (C) terminal methyl protons without alamethicin;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

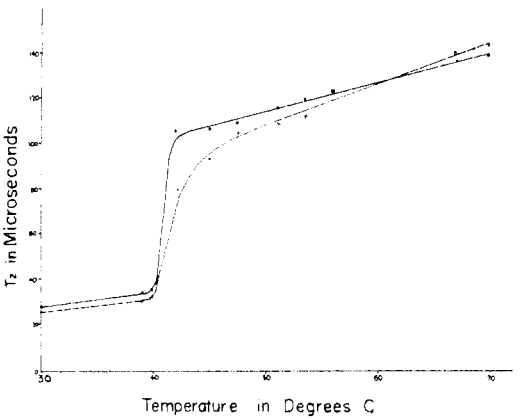

FIGURE 2: Temperature dependence of the spin–spin relaxation time of the bulk nmr signal from dipalmitoyllecithin bilayers in the absence and presence of alamethicin: solid line, no alamethicin; dashed line, with 1% alamethicin.

solution was then applied to a 200-mesh copper grid coated with parlodion sprayed with a thin film of carbon. After ~30 sec 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 ~60° to the horizontal. With the grid held horizontal, an additional drop of the phosphotungstic acid solution was then applied for 30 sec. 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 kV at mag level 12 (actual magnification on screen 100,000 and on camera 29,600).

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

Results and Discussion

Interaction of Alamethicin with Unsonicated Lecithin Bilayers. 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 nmr method. The continuous wave pmr spectrum of unsonicated lecithin bilayers above the thermal phase transition consists of two relatively sharp (100–200 Hz) signals arising from the choline methyl and the terminal methyl protons superimposed on a significantly broader (~3000 Hz) resonance due principally to the methylene protons of the fatty acid chains. This latter broad resonance obscures analysis of the sharper signals, a difficulty which 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 deadtime 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. When applied to an unsonicated lecithin bilayer, this method in essence isolates the narrower methyl signals and permits accurate intensity and line-width measurements on them. Two spin probes, located in quite different regions of the bilayer, thus become available to probe the structural and dynamical properties of the surface and the interior of the lecithin bilayer. To augment these measurements, the chain methylene protons, which account for 80% or more of the bulk nmr signal, are readily monitored by conventional pulse nmr. The utility of this combination of nmr techniques in bilayer nmr work was demonstrated in our recent study of the interaction of valinomycin with unsonicated lecithin bilayers (Hsu and Chan, 1973).

The effect of alamethicin on the crystalline $\rightleftharpoons$ 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 1 summarizes the intensities of the choline and terminal methyl proton signals as a function of temperature for lecithin bilayers containing 0 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 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 is noted that the addition of 1% alamethicin did not produce significant changes in the phase transition temperature, except that the temperature range of the transition became somewhat broadened. The addition of alamethicin did affect the choline methyl signal intensity, however. In the presence of 1% alamethicin, the intensity of the choline signal became 30–35% of the total expected intensity as compared to only 20–25% in the absence of the polypeptide. By contrast, upon the addition of 1% alamethicin, the terminal methyl proton signal exhibited no noticeable change either in intensity or in line width.

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

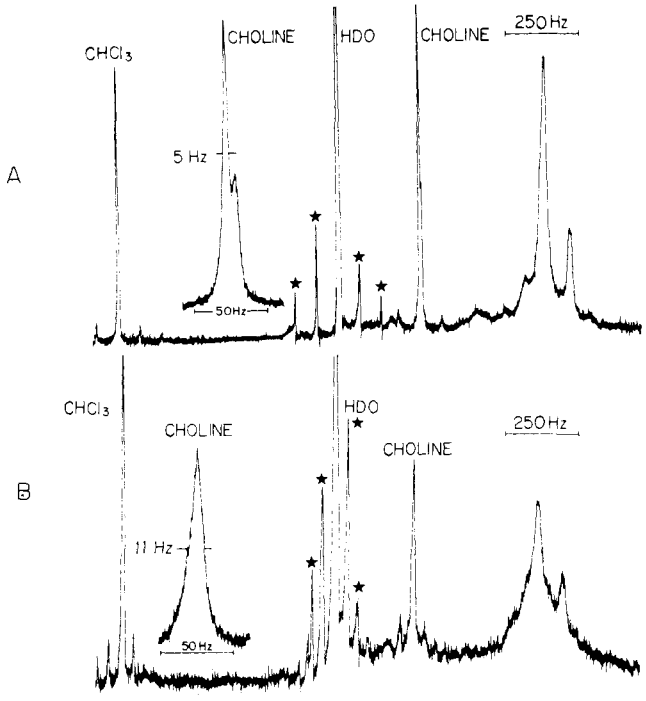

FIGURE 3: Pmr spectra (220 MHz) of sonicated lecithin vesicles at 48°. Spectrum A is for a sample containing no alamethicin, and spectrum B is for a sample containing 0.3% alamethicin. Note that the choline signal is also shown in expanded scale in both spectra. Asterisks denote spinning side bands associated with HOD signal.

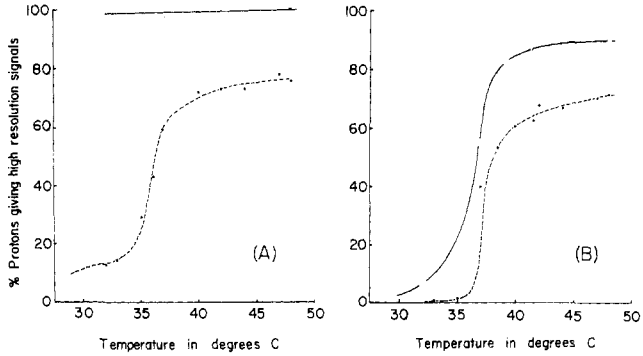

FIGURE 4: Temperature dependence of the choline methyl and fatty acid chain protons signal intensities for lecithin vesicles and vesicles incubated with 0.3% alamethicin: (A) choline methyl protons. (B) fatty acid protons. In both A and B, the solid curves give the temperature dependence in the absence of alamethicin and the dashed curves refer to the results obtained in the presence of 0.3% alamethicin.

The above observations strongly suggest that alamethicin interacts with dipalmitoyllecithin bilayer membranes principally at or near the bilayer–water interface. Although our experiments do not permit us to ascertain the various degrees to which the mobility of each individual methylene segment along the hydrocarbon chain is influenced by the polypeptide (this information could in principle be obtained using specifically deuterated or ^{13}C-enriched lecithins), we nevertheless can conclude that there is no gross overall disruption of the bilayer structure or the molecular packing of the hydrophobic interior, at least as detected by our DFT and pulse nmr experiments. It is reasonable to suggest that the alamethicin molecules are physically adsorbed onto the surface of these lecithin bilayers, and any perturbations of the bilayer structure by these adsorbed antibiotic molecules are confined essentially to the polar head group regions of the membranes. This conclusion is not unreasonable in view of the expected surface-active properties of alamethicin. That is not to say that there is no incorporation of alamethicin into the hydrocarbon region of the bilayer, but only that the partitioning of alamethicin between the hydrophobic interior and the lipid–water interface is too small to produce significant perturbations of the lipophilic regions of the bilayer.

Interaction of Alamethicin with Sonicated Lecithin Bilayer Vesicles. Freshly prepared solutions of small sonicated vesicles are colorless, nearly transparent, and homogeneous. Upon the addition of alamethicin, however, cloudiness of the sample developed within 10–15 min or longer, depending on the temperature and the relative amounts of lecithin and alamethicin in the solution. This change in the turbidity of the solution immediately suggests some alteration in the state of aggregation and/or size distribution of the bilayer vesicles. This conclusion is substantiated by nmr as well as electron microscopy experiments.

A typical pmr spectrum of small sonicated vesicles consists of two partially resolved choline signals located at ~3.21 ppm and a somewhat broader fatty acid chain signal centered at 1.27 ppm. The intensities of these resonances normally account for 80% or more of the stoichiometric concentration of protons present in the sample. The large surface curvature of small sonicated vesicles renders significantly looser packing of the phospholipid molecules and accounts for the striking difference in the pmr spectral characteristics between the sonicated and unsonicated bilayer systems. Apparently even the difference in surface curvature on the outer and inner halves of the bilayer vesicle results in different magnetic environments for the choline methyl protons.

The pmr results which we have obtained regarding the interactions of alamethicin with sonicated bilayer vesicles are essentially in agreement with those previously reported by Chapman and coworkers. Representative pmr spectra of sonicated vesicles in the presence and absence of alamethicin are shown in Figure 3. As noted earlier by Hauser *et al.* (1970), the addition of 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 in Figure 4. It is interesting to note that the observed temperature variation of the intensities for these nmr signals very nearly resembles those previously reported by Sheetz and Chan (1972) for bilayer vesicles 1000 Å in size.

Electron microscopy studies confirmed that the size distribution of the vesicles has indeed been altered with the addition of alamethicin (Figure 5). Table I summarizes the variation of this size distribution of lecithin vesicles by weight in various diameter ranges as a function of alamethicin concentration. These data are also graphically presented in Figure 6. 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% alamethicin affects the size distribution only minimally, 0.4% alamethicin significantly broadens out the size distribution. These EM 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

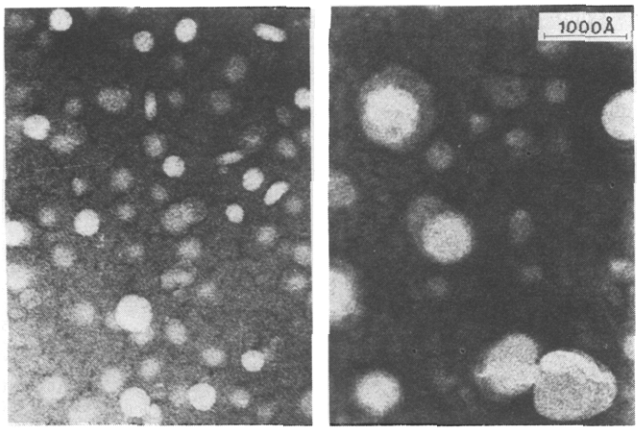

FIGURE 5: Representative micrographs for lecithin vesicle preparations: (left) sample containing no alamethicin; (right) sample containing 0.4% alamethicin.

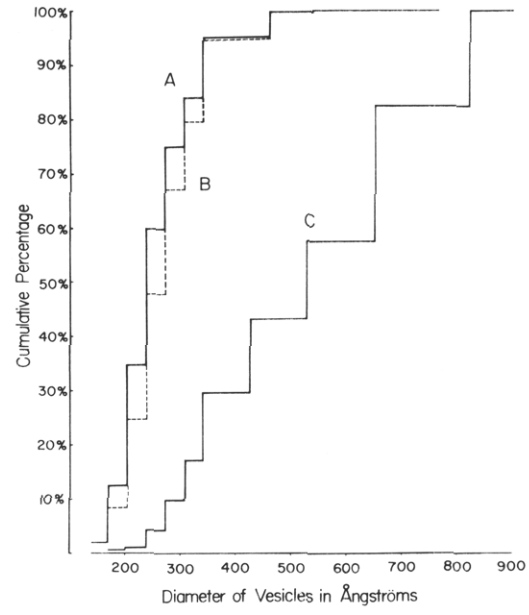

FIGURE 6: Cumulative frequency function of vesicle sizes: (A) sample containing no alamethicin; (B) (dashed line) 0.01% alamethicin; (C) 0.4% alamethicin.

0.4% alamethicin is present (Figure 7).

A comparison of the temperature dependencies of the choline methyl proton intensities of vesicles containing alamethicin (Figure 4A) with that of 1000 Å vesicles does reveal an important difference. In normal large vesicles of 1000 Å in diameter, the intensity of the choline methyl signal was previously reported by Sheetz and Chan (1972) to approach 100% of stoichiometric proton concentration when the temperature is above 40°. In the alamethicin-containing samples, only ~80% of the choline methyl intensity was observed even at 48° after the alamethicin-mediated vesicle fusion. This difference suggests that there exist specific interactions of the antibiotic molecule with the polar head groups of the lecithin bilayer and that these interactions result in immobilization of the choline methyl groups at or in the vicinity of the adsorbed polypeptide. 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 bilayers. 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 constitute further support for the notion that this antibiotic is highly surface active. Presumably when this polypeptide is adsorbed on the surface of a vesicle it can act as a nucleation center for further coagulation and fusion of the vesicles. This process will be discussed in greater detail in the following section.

Alamethicin-Mediated Fusion of Vesicles. We believe that the nmr results previously reported by Hauser *et al.* (1970) and those obtained in this work can readily be accounted for by the alamethicin-induced vesicle fusion, as well as interactions of the polypeptide with the bilayer in the vicinity of the water–bilayer interface. In this connection it should be noted that the observed increase in average vesicle size cannot be explained in terms of incorporation of the alamethicin into the hydrophobic region of the bilayer membrane, since this process in the absence of vesicle fusion could only lead to an increase of vesicle volume of about 1% at the highest concentration of alamethicin used in our work.

Fusion of vesicles occurs because small vesicles possess high surface free energy by virtue of their large curvature, and would undergo rearrangements in their molecular packing to attain a state of greater stability once this opportunity is rendered. This instability of bilayer vesicles presents an important problem and this process of fusion must be controlled before these model membrane systems can be used in reconstitution studies. For this reason the effects of temperature, ionic strength, and surface charge on the kinetics of vesicle fusion are presently under intensive investigation in this laboratory (Petersen *et al.*, 1974).

Vesicle fusion occurs when, as a result of thermal motion, they can approach each other sufficiently close 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 300-Å diameter ves-

TABLE I: Percentage by Weight of Lecithin in Different Size Vesicles in the Presence of Alamethicin.

Lecithin:Alamethicin Molar Ratio in Sample	<200 Å	200–270 Å	270–340 Å	340–420 Å	420–530 Å	530–650 Å	650–820 Å	>820 Å
No alamethicin	11	47	24	10	5	2	<0.5	<0.5
10,000	9	38	31	13	6	2	<0.5	<0.5
250	<1	4	13	12	9	14.5	25	22

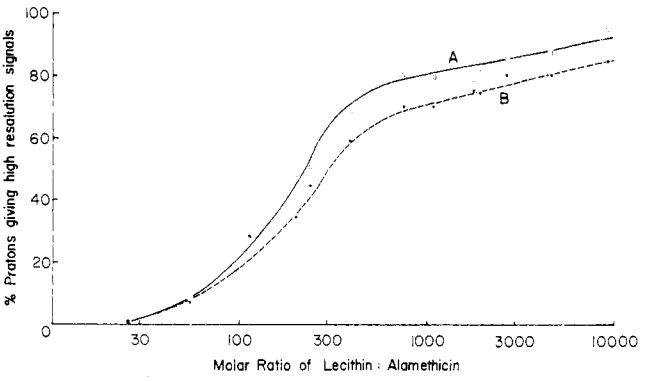

FIGURE 7: Effect of alamethicin concentration on the pmr signal intensities of lecithin vesicles: (A) choline methyl protons; (B) alkyl chain protons.

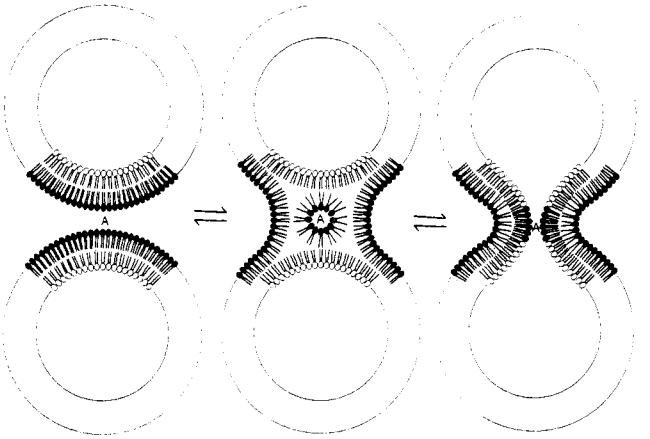

FIGURE 8: Schematic representation of the sequence of events possible for the proposed alamethicin-mediated fusion of vesicles; A = alamethicin aggregate (not to scale).

icles. Simple calculation shows that ~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 300-Å vesicles to form an 800-Å vesicle. Kornberg and McConnell (1971) have shown that the probability of outside-inside transitions of phospholipid molecules by a flip-flop mechanism is only of the order of 4%/hr, too slow to account for the rather rapid transfer of lipid molecules (~12% in less than 0.5 hr in the presence of alamethicin) from the outer to the inner halves of the bilayer.

A surface active molecule such as alamethicin might be expected to serve as a catalyst for this vesicle fusion process. This fusion can be initiated, for example, by clusters of alamethicin molecules located on the surface of these vesicles. As such clusters readily transcend the thickness of the electrical double layer of these vesicles they can serve as nucleation centers for this micelle formation. In other words, the alamethicin clusters can act as a detergent. A possible sequence of this alamethicin-induced fusion of vesicles is schematically presented in Figure 8. Termination of this process of course occurs when the external alamethicin concentration remaining in a bulk solution has been reduced to a sufficiently low concentration as a result of this endocytosis, and/or when the vesicles have grown to sufficiently large size that further fusion results in no substantial gain of stability. As one might expect, after fusion the alamethicin cluster may find itself trapped within the fused vesicle. There appears to be indirect evidence to support this. We have found, for example, that the intensities of both the choline and aliphatic signals approach zero as the alamethicin concentration is increased, an observation also previously reported by Hauser *et al.* (1970). While a total reduction in the intensity of the paraffinic signals in the high resolution spectra could arise from fusion of the vesicles alone, we feel that the result for the choline signal cannot occur without some physical interaction of the alamethicin with the polar head groups on the inner half of the bilayer. The possibility remains that there exists strong coupling between the two halves of the bilayer. However, we do not consider this alternative to be a likely one, since there is at this moment little evidence for such communication between the inner and outer halves of the bilayer in the absence of intrinsic membrane proteins.

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Studies on Motional Characteristics and Distribution of Protonated and Anionic Forms of Spin-Labeled 2,4-Dinitrophenol in Phospholipid Bilayer Membranes†

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ABSTRACT: Spin-labeled 2,4-dinitrophenol (Dnp) compounds were used to study the mechanism of action of uncouplers. From an analysis of the electron spin resonance (esr) spectra, it is possible to distinguish and measure the protonated and anionic forms of spin-labeled Dnp in phospholipid bilayer membranes. The distribution studies indicate that both forms of the uncoupler are predominately localized at the polar head-group regions of the membrane. The relevance of these findings to Mitchell's chemiosmotic coupling hypothesis of oxidative phosphorylation is discussed.

Several hypotheses have been proposed to account for the uncoupling of mitochondrial oxidative phosphorylation by various uncouplers, and they vary depending on the mechanisms of oxidative phosphorylation the authors favor (Mitchell, 1968; Van Dam and Slater, 1967; Green and Baum, 1970; Wilson *et al.*, 1971; Weinbach and Garbus, 1969). According to the chemiosmotic hypothesis proposed by Mitchell (1968), the electron transport along the respiratory chain generates a transmembrane proton gradient, which is the driving force for the enzymatic synthesis of ATP. Uncoupling agents such as Dnp[1]act as lipid-soluble proton donor–acceptor systems that dissolve in the lipid phase of the membrane and conduct backdiffusion of protons, thus collapsing the proton gradient across the inner mitochondrial membrane. Supporting evidence for such a mechanism is mainly derived from the fact that usually there is a good correlation between the ability of compounds to uncouple and their effectiveness in increasing proton conductance in phospholipid bilayer membranes (Liberman *et al.*, 1969; Hopfer *et al.*, 1968).

However, in order to provide for direct molecular evidence and to evaluate Mitchell's uncoupling hypothesis more fully, one would like to be able to measure the amounts of protonated and anionic forms of the uncoupler present in the lipid phase of the membrane. Furthermore, one would like to know the distribution and diffusibility of protonated and ionized forms of the uncoupler across the phospholipid bilayers. Toward these goals we have prepared several spin-labeled Dnp compounds so that the behavior of the uncoupler within the membrane can be directly monitored (Hsia *et al.*, 1972a,b). In the present communication, data are presented showing that it is possible to distinguish and measure the protonated and anionic forms of spin-labeled Dnp in phospholipid bilayer membranes. The distribution studies indicate that both forms of the uncoupler are predominately localized at the polar head-group regions of the membrane.

Experimental Section

Materials. Spin-labeled 2,4-dinitrophenol compounds [1-hydroxyl-5-*N*-(1-oxyl-2,2,5,5-tetramethyl-3-aminopyrrolidinyl)-2,4-dinitrobenzene, Dnp-SL(5); and 1-hydroxyl-5-*N*-(1-oxyl-2,2,5,5-tetramethyl-3-aminomethylpyrrolidinyl)-2,4-dinitrobenzene; Dnp-methylene-SL(5)] were prepared as described (Hsia *et al.*, 1972b). The chemical structures are shown in Figure 1. The p*K* values were determined by a spectrophotometric method and found to be 4.8 for Dnp-SL(5) and 5.6 for Dnp-methylene-SL(5) (Hsia *et al.*, 1972b). The spin-labeled Dnp molecules possess full activity in the uncoupling of oxidative phosphorylation in rat-liver mitochondria (Hsia *et al.*, 1972b). Egg lecithin was purchased from Pierce Chemical Co. The phospholipid was

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[1] Abbreviations used are: Dnp, 2,4-dinitrophenol; Dnp-SL(5), 1-hydroxyl-5-*N*-(1-oxyl-2,2,5,5-tetramethyl-3-aminopyrrolidinyl)-2,4-dinitrobenzene; Dnp-methylene-SL(5), 1-hydroxyl-5-*N*-(1-oxyl-2,2,5,5-tetramethyl-3-aminomethylpyrrolidinyl)-2,4-dinitrobenzene.