A DEUTERIUM NMR STUDY OF GRAMICIDIN A'

by

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Abstract

This thesis presents the results of the first application of a novel solid state nuclear magnetic resonance technique (K. P. Pauls et. al., Eur. Biophys. J. 11:1) to a naturally occurring membrane polypeptide. Deuterium NMR was used to study the structure and dynamics of hydrogen-exchanged gramicidin A*, an ion channel, in model membranes. The technique exploits recently developed procedures for solvent-signal suppression (P. T. Callaghan et. al., J. Magn. Reson. 56:101), and "depaking" powder spectra (E. Sternin, M.Sc. Thesis, U.B.C.). The spectra of gramicidin A* in crystalline form, and in the gel phase of the lipid bilayer are similar and indicate little molecular motion on the NMR timescale. In the liquid crystalline phase, however, the spectra suggest rapid uniaxial rotation of the gramicidin about the bilayer director. The frequencies of the liquid crystalline phase spectra were found to be independent of bilayer thickness, temperature, and the presence of sodium chloride, in the ranges investigated. The results are discussed in the context of the conduction properties of the gramicidin ion channel, other spectroscopic studies, and theoretical models of the structure and action of gramicidin.
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I. INTRODUCTION

The attempt to understand the biological action of proteins in terms of physical science presents a challenge to scientists of many disciplines. For integral membrane proteins this program is at the stage of structure determination. These proteins have, until recently (1,2), resisted crystallization, hence the classical x-ray diffraction techniques, so useful in studies of soluble macromolecules, have been inapplicable. The case of gramicidin A', which can be crystallized from organic solvents, illustrates a further disadvantage of the x-ray diffraction method. The x-ray analysis (3,4) of ion bound and ion free forms of gramicidin indicate a major ion-induced change in secondary structure of the polypeptide. This result is at variance with the results of spectroscopic experiments on gramicidin in artificial membranes (5). It is therefore far from certain that a given membrane protein's structure will not be affected by crystallization.

Deuterium nuclear magnetic resonance² is an effective tool for studying the structure and dynamics of ²H labelled components (for reviews see 6,7). The anisotropic rotations of a molecule in the liquid crystalline phase of a membrane partially averages the orientation dependent electric quadrupole interaction, the residual quadrupole splitting containing information about the structure and rotation of

¹Henceforth referred to as gramicidin.
²Abbreviated ²H NMR.
the molecule. Under certain conditions measurement of spectra and relaxation times can provide order parameters and correlation times for the motion of a labelled site. When the site is attached to a molecule behaving as a rigid body rotating uniaxially, with a correlation time shorter than the appropriate NMR timescale, the angle between the principle axis of the electric field gradient, and the axis of rotation may be calculated from the measured order parameter.

A synthetic polypeptide, $^2$H-labelled at the amide hydrogens, was studied using $^2$H-NMR by K.P.Pauls et al. (8). The spectrum observed in the fluid state of the bilayer consisted of a powder pattern with a single quadrupole splitting indicating a helical secondary structure for the polypeptide rotating about the helix axis, consistent with the expected alpha helix structure. Measurement of $T_2e$, the relaxation time for the decay of the quadrupole echo, allowed calculation of the rotational correlation time for a simple model of the molecule's rotational diffusion. Further, the angle between the helix axis and the amide N-D bonds was calculated from the amide $^2$H order parameter and found to be within the range predicted for the $\alpha$-helix.

The success of this study prompted application of the technique to more complicated polypeptides and proteins. Gramicidin was chosen as an interesting candidate for study because of its small size (15 amino acids), and its expected helical structure in the membrane. Despite the simplicity of
its structure, gramicidin exhibits some functionally interesting properties. In ion conduction experiments gramicidin is found to render lipid membranes selectively permeable to small monovalent cations. The conductance properties of gramicidin are well characterized; because of its large single channel conductance and other features it has continued to be of interest as a model system for biological ion transport. Furthermore, there are several groups (9, 10, 11) attempting molecular dynamics simulations of gramicidin-mediated transport. Reliable structural information is needed in this ambitious undertaking.

The results of this $^2$H-NMR study of gramicidin will be discussed along with the polypeptide study (8) and the work of K.P. Datema and K.P. Pauls (12) as well as previous spectroscopic and electrical measurements. The following major conclusions are drawn: that gramicidin adopts a fairly rigid conformation(s) which does (do) not deform to the constraints of the bilayer, and that is (are) independent of temperature in the range investigated; that no large change in secondary structure occurs in the presence of sodium ions.
II. GRAMICIDIN

A. BIOLOGICAL FUNCTION

Gramicidin, a linear polypeptide having fifteen amino acids, was originally isolated from *Bacillus brevis* (13) as a molecule with broad spectrum antibiotic action. The primary structure of the gramicidins were determined to be\(^3\):

\[
\]

where XXX denotes valine or isoleucine, and YYY denotes tryptophan, phenylalanine, or tyrosine in gramicidin A,B,C respectively (15, original references cited therein). Commercially available gramicidin A' is a mixture of valine gramicidins A,B,C in the proportions 7:1:2, and a small amount of the isoleucine gramicidins (16). Gramicidin may provide a selective advantage for *Bacillus brevis* by making a competitor's membranes leaky to cations.

Recent work (17,18) has suggested a different role for gramicidin, independent of its ion transporting action. Gramicidin-lacking mutants of *Bacillus brevis* were found unable to form normal spores unless provided with gramicidin. This effect has been correlated with the ability of gramicidin to specifically inhibit RNA transcription by inhibiting bacterial RNA polymerase. Experiments with synthetic gramicidin analogues showed that only the \(^3\)Standard three-letter amino acid abbreviations (14) are used here.
sequence:

(D-LEU-L-TRP)-Ethanolamine

is needed to restore sporulation in the mutants. As discussed below, several properties of gramicidin's primary structure, such as the strictly alternating chirality (except for glycine which is not optically active) of the component amino acids, are essential to its channel forming propensity. The author feels, then, that ion transport should not be discarded as a possible biological function of gramicidin.

B. GRAMICIDIN: A MODEL SYSTEM FOR THE STUDY OF ION TRANSPORT

Although its natural function may be obscure, gramicidin has served as an important model system in the investigation of biological ion transport. Initially found to decouple oxidative phosphorylation in mitochondria, gramicidin was characterized as an ionophore selective for monovalent cations using the then newly developed black lipid membrane technique. In a black lipid film experiment a small amount of lipid dissolved in an organic solvent is painted across a pinhole separating two compartments containing solutions of electrolytes. The lipid membrane thins until a single bilayer persists which (being much thinner than the wavelength of visible light) appears black when viewed with a microscope. Electrical properties of the membrane can then be monitored using sensitive voltage and current meters.
For bilayer forming lipids, black membranes are observed to be impermeable to ions. A crude understanding of the insulating properties may be had by considering the electrostatic self-energy, \( U \), of a sodium ion, with charge \( Q=4.8 \times 10^{-10} \) esu, and radius \( \approx 1 \) Å, in an infinite medium, given in esu units as (19):

\[
U = \frac{Q^2}{2eR}
\]

Using approximate values for the dielectric constants, \( \varepsilon = 80 \) for water, and \( \varepsilon = 2 \) for oil, we find a difference in self-energy of 3.5 eV for an ion in oil and water. The lipid bilayer thus presents a large potential energy barrier to ions.

The impermeability of the lipid matrix of a biological membrane to electrolytes has wide ranging consequences. It enables the cell to maintain an asymmetry, with respect to ion concentration for example, across a plasma or organelle membrane. This asymmetry is a universal property of biological membranes. The cells of the body, for example, have an intracellular concentration of sodium of c.5-15 mM and an extracellular concentration of c.140 mM. For potassium the extra- and intracellular concentrations, respectively, are roughly the same (20). Ion selective active "pumps" and passive "leaks" are necessary in the maintenance of a stable ion gradient. It is generally assumed that these mechanisms involve proteins embedded in the membrane, however transport activity has proven difficult to isolate (21). It is not surprising then that the ion transporting properties of
gramicidin, a simple polypeptide, excited considerable interest.

C. CONDUCTANCE PROPERTIES OF GRAMICIDIN

The conductance of ions by gramicidin has been well studied using the black lipid membrane technique described above. An elegant experiment (22) followed the time course of current fluctuations at constant voltage across a membrane containing a small amount of gramicidin. The fluctuations were found to be approximately integral multiples of a unit value, which was subsequently interpreted as the conductance of a single open channel. The autocorrelation function of the current noise was later (23) found to be an exponential indicating the channel closure to be a Poisson random process. The conductance of a single channel is independent of the thickness of the lipid bilayer(24), however channel lifetime has been found to depend on the thickness of the bilayer, the average lifetime varying between 60 seconds in a 64 Å bilayer down to .03 seconds in a 26 Å bilayer(24). Such a slow rate suggests that channel closure involves a major structural change.

The high single channel conductance observed points to a pore model for the channel, in which ions move between stationary binding sites of the channel, as opposed to a carrier model in which the ion-carrier complex diffuses across the membrane. This was corroborated by the results of (25) in which the influence of the phase of the bilayer on
transport was investigated. No discontinuous change in the conductance of a gramicidin doped membrane was observed, while a carrier doped membrane's conductance dropped radically across the liquid crystalline to gel phase transition.

Gramicidin's conductance of various ions has been measured under otherwise identical conditions (24): conductance of divalent cations and anions is negligible, and conduction of monovalent cations is (in order of decreasing conductance):

\[
H^+ > NH_4^+ > Cs^+ > Rb^+ > K^+ > Na^+ > Li^+
\]

The position of the hydrogen ion in this sequence suggests it may be transported along a file of water molecules lining the lumen of the gramicidin channel.

The bulk conductance of a membrane containing low concentrations of gramicidin was found to depend quadratically on the gramicidin concentration indicating the conducting channel as a dimer of the non-conducting species. A more detailed study of this phenomenon (26) confirmed this result, found the non-conducting species to be a single molecule of gramicidin, and showed that most, if not all, gramicidin dimers in the bilayer are conducting channels.

The equilibrium between monomer and dimer gramicidin has been found to be shifted by factors affecting the thickness of the bilayer: a transmembrane potential, and lipids with shorter acyl chains increasing the bulk conductance, anaesthetics decreasing it. An early experiment
(26) found cholesterol to decrease the bulk conductance. This may be related to cholesterol's influence on bilayer thickness.

D. STRUCTURAL STUDIES OF GRAMICIDIN

In view of its comparitively small size, the structural basis of gramicidin's biologically interesting properties presents an attractive research problem. While the conformation of the gramicidin channel has not been determined, a number of experiments have revealed some aspects of its structure, and several models have been proposed. Of these, the $\pi^{6.3}(L,D)$ helix originally proposed by D.W.Urry (28,29) (and later, independently, by Ramachandran (30)) on the basis of conformational energy considerations and model building, is the most plausible, and the only one consistent with all of the existing data.

The $\pi^{6.3}(L,D)$ helical model of the channel is a dimer of two left handed helices, having a total length of about 26 Å (28). The repeating unit of the helix is the L,D dipeptide. The amide carbonyl C-O bond directions of the L residues are towards the ethanolamine terminus, those of the D residues point in the opposite direction. This unusual alternation of hydrogen bonding directions permits head (formyl terminus) to head dimerization by completion of six hydrogen bonds, and with the alternation of alpha carbon chirality causes the side chains to lie outside the helix in sterically uncrowded positions. With 6.3 residues/turn the
helix has a 4 Å bore, and lies in a low, flat minimum on the Ramachandran plot of conformational energy versus helix dihedral angles (28). The greater number of carbonyl groups per turn of the helix than found in, say, the alpha helix and their alternating orientation could promote efficient complexing of permeant ions. The compact N-terminal formyl groups allow nearly uninterrupted structural continuity of the helix at the junction of the monomers.

Some of the conduction properties of the gramicidin channel may be understood in terms of the π̂_6'3(L,D) structure. The selectivity sequence with the channel selecting larger ions, may be due to the smaller distortions the ions produce in the helix. The pore selects cations over anions perhaps because these can be coordinated by the carbonyl dipoles lining the inside of the channel, reducing the energy barrier to transport. The coordination of ions by amide carbonyls has been demonstrated using C-13 NMR of labelled carbonyl carbons in micelle bound gramicidin(31). Thallium ion-induced chemical shifts were seen only at residues near the mouths of the channel, indicating the presence of ion binding sites. This study also ruled out the right handed π̂_6'3(L,D) helix which was inconsistent with the observed continuity of chemical shift versus alpha carbon position in the primary structure. The gating of the channel may be due to the association of monomers and the dissociation of dimers. The monotonic increase of bulk conductance with decreasing thickness is consistent with the
26 Å channel being shorter than all of the membranes investigated (24).

The \( \pi^6 \cdot 3 \) helix is consistent with the findings of several structural studies. X-ray analysis (3,4) of ion bound and ion free gramicidin showed a cylinder 32 Å long and 5 Å wide in the absence of salt, and one shorter (26 Å) and wider (6-8 Å) in the presence of salt, with 2 ion binding sites/dimer at positions consistent with the C-13 results. While these results agree very well with the \( \pi^6 \cdot 3 \) model, it has been found that the conformation of gramicidin in organic solvents (from which the crystals were grown) is quite different from the membrane conformation (5,32). As discussed below, the conformational change on ion binding probably does not occur in membrane bound gramicidin (5,32 and this study). Further, the study is at 5 Å resolution, and it is not known whether the observed cylinders are \( \pi^6 \cdot 3 \) helices.

The most convincing evidence for the \( \pi^6 \cdot 3 \) helix came from a C-13, and F-19 NMR study of labelled gramicidin in micelles (33,34). The shift reagents Tm, and Mn and a nitroxide spin label attached to DSPC were used to probe the accessibility of labels attached near the formyl and ethanolamine terminal ends to the aqueous interface and the center of the bilayer. Based on T, and chemical shift measurements it was found that the ethanolamine terminus was accessible from the aqueous interface but not from inside

\[^4\text{Distearoylphosphatidylcholine.}\]
the lipid bilayer. The opposite was found for the formyl terminus. This result eliminated other existing proposals for the membrane conformation of gramicidin.

Circular dichroism spectroscopy, sensitive to the secondary structure of biomolecules, was used to study gramicidin in sonicated vesicles (5,32). The circular dichroism of gramicidin in DMPC was found to be insensitive to the presence of a large amount (1 M) of a cesium salt, except for a minor change at long wavelengths, attributed to a small re-orientation of the tryptophan residues. The CD spectrum in DMPC was also found to be independent of temperature from 4°C to 50°C, an interval encompassing the gel to liquid crystalline phase transition of the membrane. The CD spectrum in DMPC did not depend on concentration in the range: 1:45 to 1:15 (molar ratio of gramicidin to phospholipid), however at high concentrations of gramicidin, vesicles did not form. The spectrum was found to be nearly identical in DMPC and DLPC, but in DSPC and DPPC it was markedly different. The difference in spectrum in the thicker bilayers may be due to a change in pitch of the helix or a change in equilibrium between conformations having different CD spectra. While the authors of this work favored the latter explanation, they were unable to discard the former as a possible result of change in bilayer thickness. That the spectra in DL- and DMPC are the same,

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5Dimyristoylphosphatidylcholine.
6Dilaurylphosphatidylcholine.
7Dipalmitoylphosphatidylcholine.
and exhibit no concentration dependance in DMPC, implies that one of the possible structures predominates in these membranes—the authors identifying this with the conducting channel.

The structure of the phospholipid/gramicidin system has been studied using P-31 NMR (12,35). It was found that small amounts of gramicidin induced hexagonal-II phase in bilayers of monounsaturated and saturated PC's having acyl chain length greater than 16 carbons. There was no indication of these or other non-bilayer phases in the shorter chain membranes.
III. SOLID STATE NMR AS A STRUCTURAL TOOL

Second rank tensor interactions, such as the dipolar interaction between spins, chemical shift anisotropy, and the electric quadrupole coupling are a function of the relative orientations of lab and molecule-fixed frames of reference in an NMR experiment. Measurement of the magnitude of these interactions in an oriented sample allows one to determine the directions of the principle axes of the tensor. If this information is obtained for several sites in the same system their relative orientations may be calculated.

With biological materials, an oriented sample suitable for NMR work is usually unavailable. Powder spectra, however, can yield some structural information and may be used to test existing models of structure.

An example of the application of solid state NMR techniques to the study of a membrane protein, is the $^1$C-NMR study of bacteriorhodopsin (BR) by B.A. Lewis et al. (36) $^1$C labelled leucine was incorporated into BR by adding it to the growth medium of H. Halobium. Rigid lattice and fluid phase spectra of BR reconstituted into phospholipid vesicles permitted calculation of the range of Euler angles of the leucine peptide groups with respect to the axis of rotational diffusion of BR in the membrane.
A. **DEUTERIUM NMR SPECTRA**(6,7,8,37)

The single line of an isolated deuterium nucleus' spectrum is split by the interaction of the electric quadrupole moment of the nucleus with a molecular electric field gradient into a doublet symmetric about the Larmor frequency. The quadrupole splitting $\Delta \omega$, given by the equation (8):

$$\Delta \omega(\theta, \phi) = 2\omega_q [P_2(\cos \theta) + \eta/2\sin^2 \theta \cos 2\phi]$$

where:

$$\omega_q = \frac{3}{4} \left( \frac{e^2 Q}{h} \right)$$

in which $eQ$ is the quadrupole moment of the nucleus, $eq$ is the principal value of the electric field gradient tensor, and $h$ is Planck's constant; $\eta$ is the asymmetry parameter, a measure of the departure of the electric field gradient from cylindrical symmetry; $\theta$, and $\phi$ are the spherical polar angles of the magnetic field vector in the electric field gradient principal axis system. The quadrupole splitting is usually the predominant feature of the spectrum, as other interactions, such as the dipolar coupling between deuterium nuclei, are small in comparison to $\omega_q$.

Rapid axial reorientation (compared to $\sqrt{\Delta M_2^{-1}}$, the NMR timescale) of the molecule averages the quadrupole interaction yielding a narrowed splitting of (8):

$$\Delta \omega(\beta, a) = 2\omega_q [P_2(\cos \beta) + \eta/2\sin^2 \beta \cos 2a]P_2(\cos \theta)$$

where $\theta$ is the angle between the axis of reorientation of the molecule and the external magnetic field, $\beta$ and $a$ are...
spherical polar coordinates of the axis of rotation in the principal axis coordinate system of the electric field gradient. To predict the effect of slower motions on the spectrum requires detailed calculation.

Many biological samples suitable for NMR spectroscopy are only available as "powders", consisting of all possible orientations of the molecules. The spectrum may then be computed using (6):

$$f^\pm(x) = \frac{2}{\nu} \int \sin^2 \theta \sqrt{(x \mp P_2(\cos \theta) + \eta/2\sin^2 \theta)^{-1}}\,\sqrt{(-x \pm P_2(\cos \theta) + \eta/2\sin^2 \theta)^{-1}}$$

where \(x = 2\nu/\nu_q\), \(f^\pm(x)\) are the two branches of the spectrum, which may be evaluated analytically or numerically (convoluted with the oriented lineshape) to prepare "simulated" spectra.

Axial rotation, in the short-correlation time limit, leads to a powder spectrum having zero apparent asymmetry parameter regardless of the symmetry of the electric field gradient, as may be seen by comparison of the above equations.

B. A DEUTERIUM NMR STUDY OF A SYNTHETIC POLYPEPTIDE

The synthetic polypeptide , with primary structure:

$$\text{LYS}_2-\text{GLY}-\text{LEU}_x-\text{LYS}_2-\text{ALA-AMIDE}$$

(where polypeptides with \(x = 16, 20, 24\) have been synthesized) was designed to systematically investigate lipid-protein interactions in membranes.
Figure 1 (a) $^2\text{H}$-NMR spectrum of the crystalline labelled polypeptide at room temperature. (b) Simulated spectrum with parameters $\nu_q=150$ kHz, $\eta=.16$, $T_2e=160\mu$sec. Figures from reference (8).
The 24 leucine polypeptide was the subject of a $^2$H NMR study by Pauls et al. (8) in which the labile hydrogens of the polypeptide were replaced by deuterium. The spectrum of crystalline polypeptide, shown in figure 1 (a), is well simulated (figure 1(b)) by a superposition of a broad powder pattern having parameters $\nu_q = 150\text{kHz}$, $\eta = 0.16$ due to the amide deuterons, and a motionally narrowed one with $\nu_q = 36\text{kHz}$, $\eta = 0$ attributed to rotating $^2\text{NH}_3$ groups on the lysine side chains. Except for a distortion of the narrow component, caused by a solvent subtraction procedure, the spectrum in the gel state of DPPC (figure 2 (a)) is similar to that in the crystal. This implies that the polypeptide has little mobility in the gel phase of the membrane. At 42°C, in the fluid state of DPPC, the spectrum (figure 2(b)) was found to consist of a single Pake doublet well simulated using the parameters: $\nu_q = 127\text{kHz}$, $\eta = 0$ and the measured $T_{2e} = 100\mu\text{s}$. Since the 31 amide deuterons of the polypeptide are equivalent in the spectrum, the polypeptide must assume a helical conformation with axis of motional narrowing lying along the helical axis. The splitting of the motionally narrowed spectrum allows calculation of the angle between the principal axis of the electric field gradient and the helix axis, $\beta$, to be $19^\circ$: within the range of the $N-^2\text{H}$ directions expected for the $\alpha$-helix structure. Measurement of $T_{2e}$ versus temperature exhibited a minimum corresponding to the onset of peptide rotation at the temperature of the chain-melting phase transition of the bilayer, and permitted
Figure 2. $^2$H-NMR spectrum of the synthetic polypeptide in DPPC liposomes (a) at 10°C (b) at 42°C. Figures from reference (8).
calculation of the rotational correlation time for the motion. \( \tau_c \) was found to be \( 2 \times 10^{-7} \) sec at 42°C.
IV. MATERIALS AND METHODS

A. MATERIALS
Gramicidin D (=gramicidin A') was purchased from Boehringer Mannheim and used without further purification. Dimyristoyl and Dilauryl L-α-phosphatidylcholine were purchased from Sigma Chemical Co., which quotes them as being >98% (by weight) pure, and used without further purification. Deuterium oxide (>99.8 atom %D), dimethyl-d₆ sulfoxide (>99.9 atom %D), and methanol-OD were purchased from Merck, Sharpe, and Dohme Isotopes. Gaseous ND₃ was obtained from Cambridge isotopes.

B. NMR SAMPLE PREPARATION
The labile hydrogens of gramicidin were deuterium exchanged by dissolving the gramicidin in excess methanol-OD made alkaline with gaseous ND₃, heating and stirring the solution at 50°C for an hour, then letting it sit overnight at room temperature. Bulk methanol was removed using a Buchi Rotavap evaporator; trace quantities were removed by keeping the gramicidin under vacuum overnight. The degree of exchange of the labile sites was checked using the 400 MHz proton NMR facility in the Chemistry Department. The decrease in intensity of the amide region, in the spectrum of chemical shifts, was uniform and indicated the degree of deuteration to be about 70%. The tryptophan indole sites showed a deuteration of about 35%. These results are in good
agreement with an earlier study (48).

\(^{2}\)H-NMR samples of crystalline gramicidin were prepared by simply packing the dry powder in an NMR sample tube. Gramicidin-lipid dispersions were prepared as follows: lipids and gramicidin were codissolved in methanol-OD; the methanol was removed as described above; the powdered mixture was placed in the sample tube; the desired amount of deuterium oxide added, and the sample stirred, using a glass rod, until homogeneous. Deuterated solvents were used throughout the sample preparation to prevent back-exchange the labile sites.

Typically, samples of dry, powdered gramicidin consisted of 80 mgs of the polypeptide. The total weight of most dispersions was .5 gms with: .25 gms of D\(_2\)O; .212 gms of lipid, and .038 gms of gramicidin. Thus the composition of the bilayers should be 15% gramicidin, by weight, or 1:16 moles gramicidin to moles of lipid.

C. \(^{2}\)H NMR EXPERIMENTS

\(^{2}\)H NMR spectra were recorded in quadrature at 35.5 MHz using a spectrometer built in the Physics Department electronics shop, and a Nalorac superconducting magnet. The NMR probe was tuned using a frequency sweeper. Care was taken with the crystalline samples that the coil Q was sufficiently low to avoid spectral distortion due to non-linear response of the probe. Data was accumulated on an Intel-210 microcomputer, and processed using an Intel-230 microcomputer and the
university mainframe.

The quadrupolar echo pulse sequence (50) with 3\(\mu\)sec \(\pi/2\) pulses was used with phase cycling(8), to obtain the free induction decay (FID). With some samples it was not possible to use \(\pi/2\) pulses without probe arcing, in which case 3\(\mu\)sec pulses at lower r.f. power were used.

After solvent signal suppression (40), digitization errors at the top of the echo were corrected (6), the out-of-phase component of the FID zeroed to improve the signal to noise ratio by \(\sqrt{2}\), and the in-phase component Fourier transformed.
V. RESULTS

The $^2$H NMR spectrum of crystalline gramicidin is shown in figure 3(a). The spectrum consists of a single powder pattern with quadrupole coupling $\nu_q=148$ kHz and asymmetry parameter $\eta=0.15$. The spin-lattice relaxation time, $T_1$, and the time constant for the decay of the quadrupolar echo, $T_{2e}$, were 460 msec and 170 $\mu$sec respectively. Apart from some loss of intensity in the shoulders, possibly due to the effects of the finite r.f. pulse length (38), there is excellent agreement between the experimental spectrum and one simulated using the above parameters (figure 3(b)). The absence of another inequivalent deuterium site supports the proton NMR result (see previous chapter) that approximately 90% of the deuterons in the sample are attached to amide groups. Tryptophan indole deuterons are expected to have $\nu_q=223$ kHz (39) and would be well resolved from the amide splitting. The narrow central peak in the spectrum is probably due to residual methanol-$O^2$H in the sample.

Figure 4(a) shows the in-phase component of the transverse magnetization after the second pulse of the quadrupole echo sequence for a sample containing liposomes with gramicidin. The strong, long-lived signal, due to the aqueous component of the dispersion, is superimposed on the weak, short-lived echo from the amide deuterons of the peptide. The solvent signal was subtracted following the method of Callaghan et. al. (40), the remainder is shown in
Figure 3. (a) $^2$H-NMR spectrum of $^2$H-exchanged gramicidin in crystalline form at 23°C. 100,000 transients were accumulated at a rate of 2/sec. (b) Powder pattern simulated using the parameters $\nu_q=148$ kHz, $\eta=.15$, and $T_{2e}=170$ μsec.
Figure 4. (a) Free induction decay of a sample containing DLPC, deuterated gramicidin, and D$_2$O. Discontinuities in the signal are a result of computer memory overflows. (b) Figure (a) after a fifth order polynomial, fitted to the solvent signal was subtracted. Scale: 1 cm = 49.2 \( \mu \)sec. The corresponding spectrum is shown in Figure 7 (a).
Figure 5. $^2$H-NMR spectrum of deuterated gramicidin in DPPC (a) at 20°C and (b) at 52°C. Results from Datema et al. (12).
Figure 5 (a) shows the spectrum of quadrupole splittings of $^2$H-labelled gramicidin in DPPC liposomes, recorded by Klaas-Peter Datema (12). The bilayer, at 20°C, is in the gel state and the spectrum is a single powder pattern with $\nu_Q = 144$ kHz, $\eta = 0.18$. In the fluid state of DPPC, at 52°C, the spectrum (figure 5(b)) shows a number of splittings (see Table 1) and the shape of the sides of the spectrum indicates that $\eta = 0$ for the outermost lines. Both spectra show a distortion due to the solvent-subtraction procedure, as do all of the spectra of dispersions presented in this thesis, and the region ±25 kHz was neglected in analysis of the spectra.

The fluid state spectra of gramicidin in DMPC and DLPC are given in figures 6(a) and 7(a) respectively. The splittings of the spectra are given in Table 1. Again, $\eta = 0$ for the outermost lines of the spectra. The "oriented" spectra of figures 6(a) and 7(a), shown in 6(b) and 7(b), were obtained using a "depaking" algorithm developed in this laboratory (41). This facilitates resolution of spectral lines, and permits measurement of linewidths and areas. The depaked spectra were treated to a three point binomial smoothing routine. Linewidths of the spectra were found to be in the range 8-16 kHz.

The fluid state spectrum of gramicidin in the presence of 0.1M NaCl is shown in figure 8(a) along with the depaked spectrum. An attempt to record a spectrum in the presence of
Figure 6. (a) $^2$H-NMR spectrum of deuterated gramicidin in DMPC at 38°C. 122,000 transients were accumulated. (b) Figure (a) "depaked".
Figure 7. (a) $^2$H-NMR spectrum of deuterated gramicidin in DLPC at $T = 20°C$. 167,000 transients were accumulated. (b) Figure (a) "depaked".
Figure 8. (a) $^3$H-NMR spectrum of deuterated gramicidin in DMPC at $T=40^\circ$C. The aqueous phase of the sample consisted of .1M NaCl in $^2$H$_2$O. 150,000 transients were accumulated. (b) Figure (a) "depaked".
1M NaCl was made, but the saline solution was found to decrease the coil Q-factor so much that a signal was not observable. Figure 9, the results of K.P. Datema (12), is an Arrhenius plot of $T_{2e}$ versus temperature.

| Table 1 Fluid State Quadrupolar Splittings<sup>8,9</sup>(kHz) |
|-----------------|--------------------|-----------------|-----------------|
| DLPC            | DMPC               | DPPC            | DMPC+NaCl       |
| 136 (4)         | 136 (2)            | 134             | 134 (2)         |
| 128 (2)         | 125                | 126 (2)         |                 |
| 104 (2)         | 104 (2)            | 105             | 100 (2)         |
|                 |                    | 96              | 92              |
| 70 (1)          | 67 (1)             | 65              | 70 (1)          |
|                 | 52                 | 49              | 47              |

<sup>8</sup>Uncertainty in frequencies ±1kHz.
<sup>9</sup>Numbers in parentheses are approximate relative intensities of the lines.
Figure 9 Arrhenius plot of $T_2e$ vs. temperature.
VI. DISCUSSION

In this chapter, an attempt will be made to relate the $^2$H NMR results presented in chapter four to aspects of gramicidin's structure and action.

The values for $\nu_q$ and $\eta$ measured from the $^2$H NMR spectrum of crystalline gramicidin are similar to those found for deuterons in $-N-H\ldots O=$ hydrogen bonds in many systems (39). The value of $\nu_q=148$ kHz agrees reasonably well with that calculated using the empirical relation (39):

$$\nu_q = 252.522/R(2H\ldots O)^3$$

With $R=1.8 \, \text{Å}$, expected for a $\pi^6 \cdot 3(L,D)$ helix, $\nu_q=160$ kHz. With $1.73 \, \text{Å}$, the estimated $^2$H\ldots O distance for the $\alpha$-helix, $\nu_q=150$. kHz in excellent agreement with the findings of Pauls et al. (8). It is interesting to note that the .07 Å difference in $R$ can make a significant change in frequency. This suggests that, at least in principle, the $^2$H-NMR spectrum is quite sensitive to changes in the hydrogen bonding of a molecule.

The spectrum in the gel state of DPPC is also a single powder pattern, however the parameters are somewhat different with $\nu_q=144$ kHz, $\eta=.18$. This is not surprising since, as was noted in chapter one, there is evidence that the conformations of crystal and membrane-bound gramicidin are not the same. No such change in parameters was found for

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10 Rough figure calculated assuming $-N-H\ldots O=$ colinear and using the atomic coordinates given by Koeppe and Kimura (42).
11 From coordinates given by Pauling and Corey (43), and using the assumption mentioned in footnote 10.
the synthetic polypeptide.

The fluid state spectra in lipid bilayers consisting of DPPC, DMPC, or DLPC show several lines. Wallace et al. (5) found that there was no difference between the gel and fluid state circular dichroism spectra in DLPC, DMPC, DPPC, and DSPC bilayers. We therefore attribute the dependence of the $^2$H-NMR spectrum on lipid phase to the effects of motional narrowing, rather than to a change in the structure of the polypeptide.

It is not possible to say, by looking at the spectra alone, what the axis of rotation is, or whether the rate of motion is in the short correlation time regime. Based on the discussion of chapter one, it is felt that the predominant conformation of the gramicidin is a N-terminal to N-terminal dimer, which is probably the $\pi^6 \cdot 3(L,D)$ helix. That the single channel conductance is usually stable for a channel's lifetime (24) (a timescale of seconds) suggests that the rotation of the dimer is nearly about the helix axis. Off axis motions would bury the mouths of the channel in the bilayer and prevent ion conduction.\textsuperscript{12} The rotational correlation time may be estimated using the theory presented by Pauls et al. (8) for a cylindrical molecule undergoing rotational diffusion about a single axis. With $a = 7 \text{ Å}^{13}$, $h = 30 \text{ Å}$, $\eta = 1.1$ poise (8) and $T = 40{^\circ}\text{C}$:

\textsuperscript{12} For the same reason, we expect the helix axis not to deviate much from the bilayer director.

\textsuperscript{13} An average radius estimated from a space filling model depicted in (44). The $\pi^6 \cdot 3(L,D)$ is somewhat bulkier at the C-terminal ends than at the N-terminal ends.
\[
\tau_c = \frac{(4\pi a^2 \eta)}{(kT)} = .47 \mu \text{sec}
\]
For monomers, with \( h=15 \) Å, the predicted value is \( \tau_c = .23 \mu \text{sec} \). The criterion for rapid motional narrowing is (8):

\[\sqrt{(\Delta M_2)^{-1}} >> \tau_c\]

where \( \Delta M_2 \), the change in the second moment of the motionally narrowed spectrum is:

\[\Delta M_2 = \frac{1}{5\omega_q^2} \left\{ [1 + \eta^2/15] - [P_2(\cos \beta) + \eta/2\sin^2 \beta \cos 2a]^2 \right\}\]

For the smallest splittings in Table 1, \( \omega_q = 50 \) kHz:

\[\sqrt{\Delta M_2^{-1}} = 2.6 \mu \text{sec}\]

For the larger splittings, \( \sqrt{\Delta M_2^{-1}} \) is greater.

Further evidence that the motion is in the short correlation time limit comes from comparison of the spectra recorded in differing bilayers and at temperatures in the range of 40-50°C (12). As the rotational correlation times under these conditions are surely different, the near invariance of the observed frequencies implies that the axes of reorientation are the same and that the motional narrowing about this axis is complete.

We may predict the average value of \( T_{2e}^{-1} \) (at 40°C), using the values of \( T_{2e}^{-1} \) for each line of the spectrum in DMPC calculated using the equation (8):

\[T_{2e} = \tau_c^{-1} \Delta M_2^{-1}\]

The predicted value, 50 \( \mu \text{sec} \), is, at least, the same order of magnitude as the values in figure 9.

Only two sets of deuterons, those of the D and L residues of the dipeptide repeat, are inequivalent with respect to motional narrowing about the helical axis. The
angles between the directions of the N-D bonds and the helix axis are approximately\(^1\) \(4.5^\circ\) and \(19^\circ\). Assuming that the principal axis of the electric field gradient tensor lies in the N-\(^2\)H bond direction, then the splittings of the motionally narrowed lines should be\(^5\):

\[
\nu = P_2(\cos \beta) \cdot 144kHz = 142kHz, 121kHz.
\]

As there is an equal number of L and D residues of the polypeptide the intensities of these two lines in the spectrum should be equal.

The spectra are clearly more complex than predicted above. The additional lines may arise because of the presence of non-channel gramicidin in the bilayer, or one line of the spectrum may correspond to the tryptophan indole deuterons. The two lines predicted do not coincide with any of the lines in the experimental spectrum. For the three lines, at 134, 128, 105 kHz, which have the same intensity in DMPC (and DLPC, assuming that the 128 and 136 kHz lines are not resolved), the calculated angles, \(\beta\), are, respectively:

\[
\beta = 12.4^\circ, 15.7^\circ, 25.1^\circ.
\]

Two of the lines are likely to be those arising from deuterons attached to L and D amide bonds. It is not surprising that the angles do not agree well with those predicted above, as the direction of the electric field gradient principal axis is not known and is difficult to estimate accurately. However, the discrepancy in angles may

\(^1\)Calculated using published coordinates (42) and the dimensions of the peptide bond given in (45).

\(^5\)Terms proportional to \(\eta\) are negligible.
also reflect an error in the $\pi^6\cdot 3(L,D)$ structure.

The above calculations are somewhat speculative. More definitive if less detailed statements may be made concerning the structure of gramicidin. The excellent agreement of the quadrupolar splittings in bilayers of differing thickness$^{16}$ (Table 1) and at different temperatures implies that the structure of the gramicidin peptide backbone does not deform to adapt to a hydrophobic mismatch with the bilayer. Further, the axis of rotation, as well as the molecular order parameter$^{17}$, of the conformation(s) are approximately independent of these variables in the range investigated. If the $\pi^6\cdot 3(L,D)$, or another approximately cylindrical model, is the structure of gramicidin, it seems likely that there is little tilt of the cylinder's axis with respect to the bilayer director, as this would probably depend on the constraints of the bilayer. The infrared dichroism results of Nabedryk et al. (47) suggest that the helical axis of the gramicidin channel in DMPC makes an angle of less than 15° with the bilayer normal, in agreement with our conclusions. As discussed in the first chapter of this thesis, the conductance of a single channel is independent of the thickness of the bilayer, as one would expect for a rigid pore with its axis

$^{16}$ $d$, the bilayer hydrophobic thickness, may be estimated using (46): $d = 1.75(n-1)$ (where $n$ is the number of carbons on the acyl chain). $d$ is 20, 23, and 26 Å for DL, DM, and DPPC, respectively.

$^{17}$ We may take $P_2(\cos\theta)$, where $\theta$ is the angle between the helix axis and the bilayer director, and the average is over the values of $\theta$ sampled by the molecule, weighted by the probability with which they occur.
of rotational diffusion, the axis of the cylinder, perpendicular to the plane of the bilayer.

Our results are in excellent agreement with the circular dichroism studies of Wallace et al. (5). The DMPC and DLPC depaked spectra are nearly identical, except for the 128 kHz line, which is not resolved from the 134 kHz line in the DLPC spectrum, and the circular dichroism spectra were identical in these bilayers. The $^2$H NMR spectrum in DPPC suggests there is no major change in conformation(s) in this bilayer. The large change in the circular dichroism spectrum in DPPC is thus probably due to a change in equilibrium between conformations of the gramicidin, as the authors of this paper (5) suspected.

The presence of .1M NaCl in the aqueous region of the sample was found to have little influence on the $^2$H NMR spectrum in DM and DLPC, in agreement with the CD experiments. We therefore feel that the results of Koeppe et al. (3,4) are not applicable to membrane-bound gramicidin.
VII. CONCLUDING REMARKS

This study makes it clear that the membrane structure of gramicidin is unsolved. We feel that the results presented in this thesis have deepened our understanding of the gramicidin-membrane interaction, and will contribute to the eventual solution of the structure.

This project could be extended profitably in many directions. It would be interesting, for example, to look at the effects other ions, such as Ca$^{2+}$ (which blocks the channel(49)), have on the spectrum. A $^2$H NMR study of gramicidin selectively labelled on the amino acid side chains would yield further information about the conformation.

The technique used in this work may be applied to any membrane protein amenable to hydrogen-exchange and rotating rapidly on the NMR timescale. With membrane proteins constructed of the commonest secondary structural units, the $\alpha$-helix and $\beta$-sheet, the spectra may prove to be easier to interpret than those reported here.
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