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MONODOMAIN SMECTIC LIQUID CRYSTALS
OF MEMBRANE LIPID WITH MODEL ION CHANNELS

by

Glenn Allen Olah

A thesis submitted
in partial fulfillment of the
requirements for the degree

Master of Arts

Approved, Thesis Committee:

H. W. Huang
Professor of Physics
Chairman

Harold E. Rorschach, Jr.
Professor of Physics

H. Miettinen
Associate Professor of Physics

Houston, Texas
January 1987
Monodomain Smectic Liquid Crystals
of Membrane Lipid with Model Ion Channels

by
Glenn Allen Olah

Abstract

Phosphatidylcholine multilayers containing ~24% water by total sample weight and gramicidin/lipid molar ratios, R(GL), up to 1/40 were aligned by a mechanical stressing and low temperature annealing (<70°C) technique. This technique allows large (>80μ thick x 40mm² area) monodomain defect-free multilayers containing as many as 10¹⁷ uniformly oriented gramicidin channels to be prepared. The alignment of the lipid multilayers was monitored by observing conoscopic interference patterns and orthoscopic images. The smectic defects which appeared during the alignment process were identified and dissolved. The incorporation of gramicidin in the multilayers in the form of the transmembrane channels was proven by its circular dichroism spectra (CD). A well defined CD spectrum of uniformly oriented gramicidin channels was obtained. With proof that gramicidin is in the transmembrane conducting state, it is hoped that these oriented samples will permit spectroscopic studies of the ion channel in its conducting state and diffraction studies of the channel-channel organization in the membrane. Therefore, via the oriented multilayers, a better understanding of the structure and mechanism of the gramicidin channel and other model ion channels will be possible.
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I. Introduction

Gramicidin is perhaps the best characterized ion conducting channel forming protein (Urry, 1985). It has been used as a model for studying the mechanism of transmembrane channel transport such as gating and ion selectivity (Hladky and Haydon, 1984). Also because of its known structure and properties, gramicidin can be used as a probe to study the properties of bilayer membranes (Huang, 1986). So far the experiments on gramicidin channels were primarily performed with two types of samples. The gramicidin mediated ion conductions were usually experimented with black lipid membranes, whereas the spectroscopic studies such as dielectric relaxation, nuclear magnetic resonance (NMR), Raman, circular dichroism, and infrared absorption were mostly performed with micellar or vesicular suspensions (Urry, 1985; Hladky and Haydon, 1984; Andersen, 1984). Because it was difficult to couple the spectroscopic measurements with the conduction experiments, the molecular dynamics of the gramicidin channels have not been well studied. Only a thick monodomain multilayer sample provides a bulk phase of uniformly oriented ion channels and allows spectroscopic studies of ion channels in the conduction states. Therefore, it is desirable to prepare large (80μ thick x 40mm²) samples of monodomain lecithin multilayers with gramicidin channels in the conducting transmembrane state embedded in them. Consequently, there are as many as $10^{16}$ – $10^{17}$ uniformly oriented gramicidin channels in each sample available for new types of experiments. The oriented samples now open the possibilities of studying the ion movement in the channel by
dielectric relaxation (Henze et al., 1982) and performing other spectroscopic measurements with an electric field applied along the channel. Furthermore, such a sample will allow diffraction studies of the channel-channel organization in membrane (Cavatorta et al., 1982; Spisni et al., 1983).

The purpose of this thesis is to describe the methods used for preparing oriented multilayer samples, to characterize these samples by conoscopy and polarized microscopy and to characterize gramicidin channels embedded in these oriented multilayers by circular dichroism (CD).
II. DETERMINATION OF GRAMICIDIN STRUCTURE:  
A HISTORICAL PERSPECTIVE

The gramicidins are linear pentadecapeptides first isolated from Bacillus brevis by Hotchkiss and Dubos (1941). Naturally occurring gramicidin consists of three different analogs known as gramicidin A, B, and C (GA, GB, and GC occur in the approximate ratio 72:9:19 (Glickson et al, 1972), respectively). These common analogs differ only in amino acid position 11 where GA has L-tryptophan, GB has L-tyrosine, and GC has L-phenylalanine. Also, each analog can have a variation in position 1 which can be either L-valine or L-isoleucine. In particular, the primary structure of valine gramicidin A was determined to be (Sarges and Witkop, 1964, 1965; Ishii and Withop, 1963; Hinman et al, 1950):

\[
\text{Formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-}
\]

\[
1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \quad 8
\]

\[
\text{L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NHCH}_2\text{CH}_2\text{OH}
\]

\[
9 \quad 10 \quad 11 \quad 12 \quad 13 \quad 14 \quad 15
\]

Characteristics of Val-GA to be noted are the alternating sequence of L- and D- residues, the large number of hydrophobic amino acids, and the formyl and ethanolamine end groups. Beginning in the mid-sixties gramicidin was shown to influence mitochondria, erythrocytes, electroplax, and artificial membranes by facilitating the passive transmembrane transport of alkali metal ions, protons, and water (Pressman, 1963; Chappell and Crofts, 1965; Harris and Pressman, 1967; Silman and Karlin, 1968; Podleski and Changeux, 1969; and Henderson et al, 1969). Later
investigators observed step currents or conductance changes in single membrane bilayers which were doped with minute amounts of gramicidin (Hladky and Haydon, 1970, 1972; Bamberg and Lauger, 1973). For example, with a 1.0 M KCl aqueous phase and 100mV applied voltage, the integral conductance, $\mathcal{G}$, in a glycerol mono-oleate membrane containing a small amount of gramicidin was measured to be $4 \times 10^{-11} \Omega^{-1}$ which corresponds to approximately $3 \times 10^7$ potassium ions per second per channel (Hladky and Haydon, 1970, 1972). Interestingly, gramicidin showed conductance values, like in the above example, which were typically 2 to 3 times higher than for ion-carrier antibiotics such as nonactin and nystatin. The high conductance was found to persist even when the membrane was solidified by lowering the temperature (Krasne et al., 1971). Haydon and Hladky in 1972 also found the magnitude of the conductance to be independent of the membrane thickness. Consequently, due to these and other early electrical measurements on lipid bilayers, it was proposed that ion conduction by gramicidin occurs by pore formation of two gramicidin polypeptides rather than by an ion-carrier mechanism. However, no “detailed” structural information about gramicidin incorporated in the lipid bilayer was ascertained in these early conductance experiments (Hladky and Haydon, 1984).

In 1971, based on a conformational energy analysis, Urry proposed a single-stranded, lipophilic, left-handed helical structure for gramicidin A in which the C-O bonds alternately point toward the amino and carboxyl ends. The alternating C-O directions allow hydrogen-bonded dimerization by a formyl-to-formyl attachment so that two of these helices can combine
to form a transmembrane channel; see figure II-1,(a). The intramolecular hydrogen-bonding pattern of this helical structure resembles the antiparallel β-pleated sheets. Initially the only strong evidence to support this model was the inactivity of desformyl gramicidin A in ion-transport studies (Urry; Urry et al., 1971). After Urry proposed this single-stranded dimeric model, it was hoped to find an organic solvent in which gramicidin adopted the same conformation as in the membrane bound state. If an appropriate organic solvent could be found then spectroscopic characterization of the membrane bound state could easily be done. However, upon spectroscopic investigation of gramicidin in various organic solvents, it was found that gramicidin could adopt a variety of different conformations (Urry et al., 1972; Veatch et al., 1974a,b). In dioxane alone, four conformational species were physically separated by thin-layer chromatography (Veatch et al., 1974a). Circular dichroism and infrared spectrums of these various gramicidin conformers in dioxane indicated that, besides the possible single-stranded dimeric conformation, one of the species is possibly a double-stranded β-helix formed by intertwining two (L,D) peptides in a parallel or antiparallel fashion (Veatch et al., 1974a,b); see figure II-1,(b). This second type of helical structure also appeared to be a stable candidate for the transmembrane channel.

Before the transmembrane conformation was narrowed down to either the single- or double-stranded helices, it was shown that the membrane-bound channel was indeed the result of a dimerization reaction. A large conglomeration of evidence had established the dimer as the "fundamental" unit necessary for conductance:
1) The earliest support for a dimer as constituting the smallest conducting unit was the dependence of membrane conductance on the 2nd power of gramicidin concentration in solution (Tosteson et al., 1968).

2) A covalently linked dimer, malonyl-bis-desformyl gramicidin A', showed similar channel activity as gramicidin A' (Urry et al., 1971; Bamberg and Janko, 1977)\(^1\).

3) Relaxation kinetic analysis of transmembrane conductance response to a voltage jump is consistent with the assumption that equilibrium exists between nonconducting monomer species and the conducting dimer.

4) From autocorrelation analysis for gramicidin A-doped lipid bilayer membranes, measurement of the reciprocal value of the dissociation rate constant \(k_d\) under a variety of conditions was shown to be practically equal to the mean channel lifetime \(\tau^\ast\). This result is in agreement with the dimerization model of channel formation (Kolb et al., 1975; Kolb and Bamberg, 1977)\(^2\).

5) Simultaneous fluorescence measurements of planar bilayer membranes containing a highly active and fluorescent analog of gramicidin A, dansyl gramicidin C, gave a slope of 2.0 ± .3 for the plot of the logarithm of the conductance versus the logarithm of the fluorescence intensity (Veatch et al., 1975).

6) Conductance and fluorescence energy transfer studies of hybrid

---

1 Gramicidin A' refers to the commercially available gramicidin which is predominantly gramicidin A.

2 Bamberg's group also used relaxation kinetics to determine the dissociation rate \(k_d\). The dimer association was also concluded.
channels formed from two different gramicidin analogs support the dimerization model (Veatch and Struder, 1977).

7) Recently, a laser temperature-jump technique was used to measure the current relaxation time of gramicidin A doped lipid bilayer membranes. The results were identical to that reported for V-jump experiments as done by Bamberg's group (Stark et al., 1986).

All of the above results established with strong certainty that the dimer was indeed the "fundamental" conducting unit. However, the possibility of dimer-dimer aggregation into tetramers or higher oligomers was disregarded. In other words, it was not conclusive if dimers aggregate into tetramers, hexamers, etc. Actually, based on conformational studies, Urry considered the possibility of dimer-dimer interaction as early as 1972 (Urry, 1972b) and recently our research group examined aggregation effects by considering the minimization of the lipid bilayer deformation free-energy in the presence of gramicidin A' (to be published, 1987). Other investigators also showed evidence for dimer aggregation in lysolecithin suspensions based on electron microscopic and fluorescence studies and concluded the intermolecular interaction to be due to Trp-Trp interaction (Cavatora et al., 1982; Spisni et al., 1983).

From 1975 to 1982 a large amount of evidence finally established the formyl-to-formyl joined single-stranded β-helix originally proposed by Urry as the dominate transmembrane channel structure. Support for this model resulted from studies where gramicidin A was chemically modified at or near the formyl or ethanolamine end groups to see what effects the
modifications had on the bilayer conductance activity. In particular the changes in the 9th and 11th residues had minor effects on the channel lifetime, $\tau$ (Bamber and Benz, 1976). Similarly, analogs modified at the ethanolamine end showed little alteration of the values for $\gamma$ and $\tau$; however, even the slightest modification at the formyl end resulted in dramatic changes in channel activity (Apell et al., 1977; Bamberg et al., 1977; Bradley et al., 1978; Szabo and Urry, 1979; Pradad et al., 1982a,b, 1983). These chemical modification studies coupled with conformational and ionic transport energetic studies enabled researchers to conclude the structure to have 6.3 residues per turn. Unfortunately, these studies did not illuminate on whether the helical structure was left- or right-handed.

Beginning in the mid-seventies, nuclear magnetic resonance (NMR) and other spectroscopic techniques allowed characterization of gramicidin incorporated in phospholipid vesicles suspended in water. In 1975 Veatch et al. demonstrated that the majority of dimerized gramicidin are ion-conducting channels and later in 1977 Veatch and Stryer, by using fluorescence energy transfer measurements, showed that gramicidin is indeed dimerized in phosphatidylcholine vesicles. Hence by utilizing $^{13}$C and $^{19}$F NMR and attaching appropriate labels to the gramicidin termini, measurements of both $T_1$ chemical shifts and spin lattice relaxation were used to determined the accessibility of these labels to paramagnetic probes placed in the aqueous and fatty acid chain regions (Weinstein et al., 1979). This study indicated that the orientation of gramicidin in these phospholipid vesicles was such that the ethanolamine end was close to the surface of the membrane while the formyl end was buried deep within the
bilayer structure thus directly supporting the Urry model (Weinstein et al., 1979). The vesicle-packaged gramicidin was further characterized by comparing the circular dichroism (CD) spectra of various gramicidin analogs and derivatives in vesicles with their conductance activities found in transport studies (Wallace et al., 1981). In dilauryl- and dimyristoyl-phosphatidylcholine vesicles, a single unique CD spectra was found only for the highly conducting analogs (Wallace et al., 1981).

In an independent study from Weinstein et al., Urry et al. characterized an aqueous gramicidin-lysolecithin system. This group first gathered proof that the gramicidin was actually in the transmembrane state. This was done by noting that the transmembrane state requires the gramicidin to span the hydrophobic region of the bilayer. Using $^{13}$C NMR and adequate heat incubation ($-70^\circ$C for 15hrs) of the suspension, the mobilities of the aliphatic lipid carbons of egg yolk L-α-lysolecithin were shown to be remarkably reduce when gramicidin was present (Urry et al., 1979a). This indicated that gramicidin was indeed incorporated in the lipid component of the micellar structures. In the time course of heating, the CD spectra of gramicidin went through dramatic changes. A stable and unique CD spectra was found for the gramicidin after appropriate heating. This unique spectra was the same spectra as found for the conducting gramicidin analogs in the DLPC and DMPC vesicle suspensions. Further, $^{23}$Na NMR longitudinal relaxation, $T_1$, studies indicated that only this unique and stable state had significant sodium ion interaction which implied channel occupancy (Urry et al., 1979a,b). Once ion interaction was observed, a NMR deduced energy of activation for the exchange of the
sodium ion with the channel binding site gave values ranging from 6.8-7.4 kcal/mole (Urry et al., 1979a, 1980). This compared favorably with the energy of activation measurements done on black lipid membranes where values from 5 kcal/mole for glyceryl monooleate -decane membrane (Hladky and Haydon, 1972) to 7.3 kcal/mole for dioleoyl lecithin membrane (Bamberg and Lauger, 1974) have been reported. Once gramicidin was shown to be in the transmembrane state, measurements of carbonyl carbon shifts induced by ion interaction with the channel again singled out the Urry model as the correct channel conformation (Urry et al., 1983). These studies even indicated a left-handed helicity for the channel (Urry et al., 1982). (A full account of NMR conformational analysis of gramicidin is given in Urry's 1985 review.)
FIG. II-1. (a) The single-stranded β-helical dimer formed by formyl-to-formyl attachment of two left-handed (L,D) gramicidin A peptides as proposed by Urry in 1971. The conformation shown has 6.3 residues per turn and a channel length of 28Å. (b) The double-stranded β-helical dimer formed by intertwining two (L,D) peptides in an antiparallel fashion as proposed by Veatch in 1974. There are 5.6 residues per turn and a channel length of 32Å. The circle labeled M represents a monovalent cation moving in the channel. (The hydrogen bonds and the amino acid side chains are omitted for clarity.) [From Ivanov and Sychev. The gramicidin A story. In "Biopolymer Complexes" (G. Snatzke and W. Bartmann, eds.), pp. 107-125.]
III. MATERIALS AND ANALYTICAL METHODS

1. Materials

Phosphatidylcholines: DLPC (lot #64F-8375), DMPC (lot #105F-8460) and DPPC (lot #55F-8350) were purchased from Sigma Chemical Company. All three lipids were synthetically produced. Lipids were always stored in the dark, below -0°C, and under dry nitrogen.

Gramicidin was obtained from Sigma Chemical Company (lot #84F-1087) and from ICN Pharmaceuticals (lot #19497).

Deuterium oxide (lot #35F-3432) was purchased from Sigma Chemical Company. D₂O was stored in a desiccator at room temperature.

The surfactant DMOAP (lot #44009), 50% in methanol, was purchased from Petrarch System Inc., Bristol, PA.

Fiske-SubbaRow reducing agent (lot #11F-70709) and acid molybdate solution (lot #65F-6154) were purchased from Sigma Chemical Company and stored at room temperature in the dark. These reagents were used to determine the phospholipid concentrations.

Silicone rubber was purchased from Specialty Silicone Fabricators, Inc., Paso Robles, Cal. Mylar, .06, .48, .75, .92, 1.42, and 2.0 mils thick, were supplied by DuPont. Mylar, .12 and .24 mils thick, were bought from SPEX Industries, Inc., Edison, N.J. Silicone rubber and mylar were used as spacers which sealed and determined the thickness of the multilayer samples.
Substrates: highly polished ophthalmic crown glass plates, $n_D=1.523$, and commercial grade fused silica plates were supplied by Optical Instruments in Houston, TX.

Only singly distilled/deionized water with resistivity greater than 2MΩ/cm was used. All solvents were of high commercial grade and run through a Buchner filter with 40-60μm pore sizes before use. Gramicidin and DMOAP surfactant were refrigerated and stored in the dark.

2. **Analytical Methods**

In this section, the analytical method used to determined the purity of the lipids and amino acid analysis of commercial gramicidin are described. Methods for determining the peptide and lipid concentration for both the multilayer and the vesicle preparations are also discussed. Gramicidin in phospholipid vesicle suspensions were prepared so a comparison with the circular dichroism spectra of gramicidin in aligned multilayers could be made; hence, the vesicle preparation is described. A brief description of the alignment technique for the multilayers is given and will be elaborated on in chapter IV.

Lipid Purity

Thin-layer chromatography was used to check the purity of the commercial phosphatidylcholines. This purity check was done on all lipids immediately after opening the vial. Also, samples containing lipid which were sealed and stored under dry nitrogen and then left at room
temperature for more than 1 week were reassessed by this method before use. Silica gel 6 plates (5 x 20 cm, 250 μm) from Anaitech along with an elution solvent consisting of chloroform/methanol/water in a volume ratio 65/25/4 were used. The chromatograms were developed by placing them in a container lined with iodine thus exposing the plates to iodine vapor. This method allows phospholipid purity to be tested to ~1% therefore only phospholipid found to be greater than ~99% pure were experimented (Powers and Pershan, 1977).

Amino-acid Analysis

High pressure liquid chromatography, HPLC, was used to determine the analog makeup of the commercial gramicidin. The gramicidin, dissolved in methanol, was run on an ES Industries, Chromegabond C8 reverse phase column (4.6 x 250 mm, 5μm beads). The column was eluted with 80% methanol at 1.5ml/min. Gramicidin (lot #19497) from ICN Pharmaceuticals was shown to have a mixture of gramicidin A, B, and C given by the ratio 73:7:20, respectively; see figure III-1, (a). Similarly, gramicidin (lot #84F-1087) from Sigma Chemical was shown to have a mixture ratio given by 70:8:22; see figure III-1, (b). These analog ratios are comparable to that given in the Merck Index and in other literature material (eg., Glickson et al., 1972; Gross and Withop, 1965).

3 If the sample contains gramicidin and this volume ratio is used for the elution solvent, then the gramicidin will elute along with the solvent front since the polypeptide is so hydrophobic.
Gramicidin and Lipid Concentration

The protein concentration was determined spectrophotometrically. In the case of the multilayer samples, a small hole with a known volume, .0105 ml, was cut in a flat aluminum block. The sample mixture was placed in the hole and a glass cover slide was slid over the top of the hole so as to level off any excess sample amount. The hole was .762mm deep and 4.19mm in diameter. These dimensions were such that the error due to the bowing of the sample mixture at the surface was small. In view of the fact that the lipid can readily exchange water with the atmosphere, the volume measuring was done as quickly as possible, typically taking less than 5 seconds. The sample mixture was then scraped out of the hole and dissolved in 2 ml of methanol. Using an extinction coefficient of 22,500 mol⁻¹cm⁻¹ at 281nm for gramicidin in methanol, the peptide concentration was determined by using a Cary 17 spectrophotometer. In the case of a vesicle suspension, a few milliliters of the suspension were removed and lyophilized. Once all of the water was removed, the remaining sediment was dissolved in 2 ml of methanol and the peptide concentration was determined by UV absorption as above.

A modified Fiske-SubbaRow method (Dittmer and Wells, 1969; Fiske and SubbaRow, 1925) was used to determine the lipid concentration. The same method was used for both the multilayer liquid-crystals and vesicle suspensions. Approximately 100-150 μl of the 2ml methanol solution (prepared in the protein concentration measurement) was placed in a 15 x 150 mm pyrex test tube. The methanol was evaporated off and 150 μl of
70% perchloric acid was added to the mixture. Since lipids rapidly oxidize under acid reflux, with quantitative release of phosphorus as inorganic phosphate, the sample was heated with a Bunsen burner to allow acid condensation and reflux. Digestion of the lipid was continued until the sample color cleared, typically taking about 10 minutes. After digestion, the sample was cooled to room temperature and 5 ml of distilled water and 1 ml of an acid molybdate solution (ammonium molybdate-4H₂O, 1.25g/dL, in 2.5 N sulfuric acid) were added. The sample was thoroughly mixed with a vortex mixer. The ammonium molybdate reacts with the inorganic phosphate to form phosphomolybdic acid. Phosphomolybdic acid was reduced by adding 150μl of Fiske & SubbaRow reducing agent (1-amino-2-naphtol-4-sulfonic acid, 0.8%, sodium sulfite and sodium bisulfite). The sample was mixed by inversion and allowed to stand for 10 minutes. Using a Cary 17 spectrophotometer, the inorganic phosphate concentration (proportional to lipid concentration) was determined by reading absorbence at 660nm relative to a reference blank. The blank was prepared along with the sample aliquot and consisted of the same ingredients as the sample except minus the sample sediment. Also, before the above procedure was used, a standard calibration curve was prepared. A slope of 0.0178 ± 0.0002 μg⁻¹ was found for a standard plot of absorbence versus inorganic phosphate for a 1cm pathlength.

Vesicle Preparation

The gramicidin containing vesicle suspensions were prepared in a similar manner as described by Weinstein et al. (1979). The appropriate
amount of polypeptide and lipid (~200mg), gramicidin/lipid molar ratios, R(G/L), between 1/40 to 1/300, were dissolved in CHCl₃. The CHCl₃ was rotatory-evaporated off and the solutes were spread as a thin-layer onto the wall of a 100 ml round-bottom flask. The sample mixture was further left under a vacuum (<10 μ) for 2 hours to ensure total solvent removal. Approximately 10 ml of H₂O was added to the flask and incubated at 30°C so as to hydrate the sample. The sample was transferred to a cup horn accessory attached to a Branson sonifier model 185. The sample was flushed with nitrogen and then sonicated at power 5 for 0.5 hours at 42°C. The sample was removed from the cup horn and diluted to a final lipid concentration of 4 mg/ml. Finally, to ensure minimal light scattering, the diluted vesicle suspension was further extruded through polycarbonate membrane filters in decreasing pore sizes of .4μm, .2μm, .08μm, and .05μm. An aliquot of the suspension was taken for the peptide and lipid concentration determination. The CD spectra were then measured as described in chapter Ⅳ.

Multilayer Alignment

The multilayer sample alignment was monitored by observation of interference patterns under a conoscopic or observation of orthoscopic images under a transmission polarizing microscope. The conoscopic optical setup is shown in figure III-2. An Oriel model 79000 2mW helium-neon laser, λ=632.8nm, was used as a light source. The laser source had a beam divergence (total angle) less than 1.2 mrad and a polarization ratio ≈ 10⁻³. Three different types of glass were used in the
optical system: 1) quartz plates, \(n_{632.8\text{nm}} = 1.457\) and \(1\times2\times1\text{mm}\), were used to sandwich the samples, 2) Nikon camera polarizer filter between glass with \(n_{632.8\text{nm}} = 1.509\) and \(0.229\text{cm}\) thick was used as the conoscope analyzer, and 3) ophthalmic crown glass, \(n_{632.8\text{nm}} = 1.521\) and \(4.32\text{cm}\) thick, was used for the remaining glass components of the system. A schematic diagram of the orthoscopic optical setup is shown in figure III-3. A trinocular Labophot-Pol Nikon polarizing microscope was used as an orthoscope.

An isotropic sample transmits light with equal velocity in all directions (index of refraction: \(n_x = n_y = n_z\)). Under a conoscope or polarizing microscope\(^4\) with crossed polarizers, this sample appears completely dark\(^5\). On the other hand, an uniaxial crystal sample \((n_x = n_y = n_z)\) can produce a definite conoscopic interference pattern (in this case, the \(z\)-axis is known as the crystallographic \(c\)-axis). If the \(c\)-axis of a crystal coincides with the cylindrical symmetry axis of the conoscope optical system shown in figure III-2, then the crossed polarizers will only extinguish transmitted light for specific propagation directions. The resultant optical picture on the screen will consist of alternating dark circles and illuminated circles that grade around in both directions from the 45-degree azimuths into a more or less diffuse black cross. See figure IV-10. Also, a crystal with its \(c\)-axis parallel to the line of sight of the

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\(^4\) Polarizing microscope refers to the orthoscope optical setup shown in fig. III-3.

\(^5\) An isotropic cross is commonly seen under the conoscope with crossed polarizers even when no sample is present. This pattern owes its origin to slight rotation of the vibration of the linearly polarized light from the laser as it is incident on the curved surfaces of various lenses in the microscope objective.
polarizing microscope produces no path difference in the transmitted light so the image remains black or gray between cross polars even during a complete rotation of the crystal about the optical axis. A biaxial crystal transmits light with refractive indices unequal in all directions \( (n_x \neq n_y \neq n_z) \). Under a conoscope, in which the acute bisectrix coincides with the cylindrical symmetry axis\(^6\), the interference pattern consist of a hyperbola encircled by Cassini ovals. Finally, for the conoscope, the light which hits a point on the screen can usually be traced back to a small region in the sample through which the light traveled. Therefore, a multidomain sample will have a mosaic conoscopic interference pattern when viewed between crossed polarizers.

A full account of the optical properties of crystals can be found in "The Principles of Optics" by Born and Wolf.

\(^6\) A biaxial crystal contains two optical axis and the optic axial plane is defined as the plane that contains both of these axis. The acute bisectrix refers to the axis in the optical axial plane that bisects the two optical axis of the crystal.
FIG. III-1. HPLC resolution of the six gramicidin found in two different lots of commercial gramicidin. The gramicidin, was dissolved in methanol, and run on a ES Industries, Chromagabond C8 reverse phase column (4.6 x 250 mm, 5 μm beads). The column was eluted with 80% methanol at 1.5ml/min. The peak assignment for both graphs are given by the following: A1, valine gramicidin A; A2, isoleucine gramicidin A; B1, valine gramicidin B; B2, isoleucine gramicidin B; C1, valine gramicidin C; C2, isoleucine gramicidin C. (a) Commercial gramicidin (lot #19497) from ICN Pharmaceutical. (b) Commercial gramicidin (lot #84F-1087) from Sigma Chemical.
FIG. III-2. A schematic diagram of the conoscope. The polarizers are crossed and the microscope objective (100x) focuses the laser light (convergent light) approximately .5 mm above the sample. Therefore, a cone of light intercepts a small region of the sample producing an interference pattern at the screen.
FIG. III-3. A schematic diagram of the polarizing microscope used as an orthoscope. Shown is a set of parallel inclined light rays which are passed through the lower polarizer and focused on the sample. The light then passes through the upper polarizer which is crossed with respect to the lower polarizer. Before the light comes to a focus to form an image, it is brought to a focus in a plane which includes a calibration hair scale. The light passes through the ocular lens and a image is finally focused on the retina of the eye.
IV. ALIGNMENT OF PHOSPHOLIPID-WATER MIXTURES WITH AND WITHOUT GRAMICIDIN

1. Introduction

This chapter describes the techniques used for preparing large monodomain smectic liquid crystals, LC, made from lipid-water mixtures which may or may not contain other biological components such as gramicidin. (If other biological ingredients are present, then the lipid-water LC serves as a matrix to support these components.) In general, the techniques described include mixing of the lipid and other ingredients, hydrating the lipid component, treating the glass substrates so as to induce homeotropic alignment (smectic layers parallel to the glass surface; see figure IV-1), and aligning of the mixture by using an annealing/mechanical method.

Before a thorough discussion of the preparation techniques proceeds, a brief description of the various liquid crystal defect structures which appear during the alignment process should be considered. This will allow one to judge the degree of alignment attained. In order to understand LC-defects, the phase behavior of lipid and lipid-water systems will first be expounded upon.

Anhydrous phospholipids exhibit thermotropic mesomorphism; in other words, the lipid does not pass directly from a solid to a liquid when heated. At low temperatures the PC will show signs of birefringence. On heating, three processes occur: 1) some loss of birefringence at a first transition temperature ($\approx$120°C for DMPC), 2) a small increase of
birefringence (≈135°C for DMPC), and 3) finally a pronounced overall loss of birefringence near the melting point of 230°C (Byrne and Chapman, 1964; Chapman and Collin, 1965). The first process gives way to a "melting" of the hydrocarbon chain region of the PC and the second process is similar to that which occurs with liquid crystals, such as ρ-azoxyanisole or cholesteryl acetate which form nematic and cholesteric liquid crystalline phases, respectively. The third process corresponds to the breaking of the ionic linkage associated with the polar groups of the PC and is carbon chain length independent.

Figure IV-2(a,b) shows the phase diagram of a DPPC-water system (Chapman et al., 1967; Powers and Pershan, 1977). The lipid-water systems exhibit lyotropic mesomorphism; in other words, the phase behavior of lipid-water systems is concentration dependent (in this case, dependent on the degree of hydration). These lyotropic phases still shown thermotropic mesomorphism, which simply means the phases depend on both temperature and concentration. The thermotropic phase transition temperature, T1, shown in figure IV-2(a) can be understood if the water is seen as diffusing into the polar (ionic) region of the PC crystals. This penetration of water affects the whole crystal structure and causes a reduction of the dispersion forces in the hydrocarbon chains. The transition temperature has been shown to correspond to the "melting" of the hydrocarbon region but it also depends on the simultaneous weakening

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7 Similar phase transitions occur for all the saturated phosphatidylcholines.

8 "Melting", in any context, means the region in question becomes liquid-like with a large increase in mobility.
of the hydrocarbon chain interaction caused by the water (Chapman, 1975). At high water content (≈ 40wt% or greater), the transition temperature has a lower limit, $T_C$, which corresponds to the minimum temperature required for water to penetrate between the layers of the lipid molecules. The penetration of water will cause the bilayers to form structures other than the planar lipid bilayers separated by water such as vesicles or hexagonal structures. The region labeled gel, if attained by lowering the temperature from the mesomorphic lamellar phase, will be a state with crystalline paraffin chain regions which may or may not be metastable. If it is metastable, then the system will transform into micro-crystals of amphiphiles in water which are stable and independent of the samples thermal history.

The mesomorphic lamellar regions labeled $L_\alpha$ and $L_{\alpha'}$ in figure IV-2(b) are referred to as smectic A in the liquid crystal literature (De Gennes, 1974). As shown from X-ray diffraction studies and confirmed by our preliminary neutron scattering (unpublished), these regions consist of planar lipid bilayers separated by water layers whose thickness depends on water concentration (Levine et al, 1968; Powers and Clark, 1975; Chapman et al, 1967). See figure IV-1. The detailed difference between $L_\alpha$ and $L_{\alpha'}$ is not yet clear. Homeotropic alignment of lipid-water systems by using the annealing/mechanical method requires the sample to be in the smectic A phase. It is in this phase that the lipid can acquire enough flow during mechanical stressing to allow defects to surmount the energy barriers necessary to form aligned domains. In particular, the defect structures associated with the smectic A phase need to be described.
The various smectic A defects found in lipid-water systems are fairly well understood. Friedel and Grandjean were the first to recognize that the energy required to change the distance between layers in a lamellar phase is much greater than that required to bend layers. Hence they proposed focal conic defect structures which preserve an uniform layer spacing where the layers can form Dupin cyclides with line discontinuities lying on a hyperbola or ellipse. See figure IV-3. This type of defect is common in many smectic systems; however, it is only occasionally present and not the most common defect in the lipid-water lyotropic system. The most common defect is the undulating paired disclination or "oily streak". As shown in figure IV-4, the "oily streak" defect does not necessarily maintain uniform spacing between adjacent layers as for the focal conics. This structure could be viewed as two equal and opposite dislocations with large Burgers vectors (Kleman et al., 1976; Asher and Pershan, 1979b; Benton and Miller, 1983; Schneider and Webb, 1984). Schneider and Webb give an excellent optical analysis of the "oily streak" in thin, fully hydrated lyotropic LC of DMPC. Other common defects appear as birefringent polygonal arrays under a polarizing microscope with crossed polarizers. The least birefringent of these polygonal structures have been labeled parabolic focal conics, PFC's (Rosenblatt et al., 1977; Asher and Pershan, 1979a,b). See figure IV-5. The more birefringent arrays are more complicated than the PFC's and thus not well understood. While the PFC's readily anneal away when the sample temperature is slowly lowered, the more complicated arrays occasionally pose a problem. Defects other than "oily streaks" and polygonal arrays are less common if care is taken during
the preparation and alignment processes. Some of these other defects encountered result from lowering the temperature too fast such as strandlike defects (Kleman et al, 1976; Asher and Pershan, 1979b). See figure IV-6. Others result from stressing the sample too much such as highly birefringent globular areas which grow and eventual diffuse into "oily streaks" under continued stressing. See figure IV-7. Dirt and dust impurities in the sample can also be a problem. Dust particles seem to get trapped in the "oily streaks" and usually can be mechanically swept out of the sample but not always. Dust has also been seen lodged in the core of the PFC structures. Large (up to 80μm thick x 40mm²) well-aligned monodomain LC are achieved only after all the mentioned defect structures are removed.

2. Sample Preparation

In this section, methods for preparing lipid-gramicidin-water mixtures are described. These mixtures are to be aligned by the mechanical/annealing method which is discussed in a later section.

Mixing Lipid and Gramicidin

Gramicidin and lipid (typically 200mg), in molar ratios of 1/40 to 1/300, were dissolved in CHCl₃ in order to thoroughly mix the components. The mixture solution was rotary-evaporated so as to remove the solvent (CHCl₃), and the solutes were spread as a thin-layer onto the wall of a 100ml round-bottom flask and then dried under vacuum (<10μ) for 2 hours. The flask was flushed with dry nitrogen and a small amount (~1ml/gm) of
benzene was then added. The solution was incubated at 37°C until all the solute dissolved. If the lipid was not completely dry then a suspension is formed in the benzene, not a solution. In this case, a higher incubation temperature may help (not to exceed 70°C) or a small amount of methanol can be added. The solution was quickly frozen (in a dry ice and isopropanol bath) and placed under vacuum (<10μ) for 24 hours. After lyophilization the mixture was allowed to reach room temperature and it was further left under vacuum for another 48 hours to ensure total removal of the benzene solvent. We found that samples which were lyophilized from benzene were easier to align and had fewer defects than samples prepared by more direct means. This is possibly true because lipids dissolved in benzene are thought to form large inverse micelles that have a high degree of local order (Asher and Pershan, 1979; Elworthy, 1959, 1960). Also, the samples which contained gramicidin were consistently more difficult to align than those without gramicidin.

Sample Hydration

The lipid-gramicidin mixture must be in the smectic A (or Lα) phase before it can be aligned; therefore, it must be hydrated to some degree and

9 Phospholipid is extremely hygroscopic, therefore, the lipid must be quickly weighed and dissolved in CHCl₃ in order to prevent undesired hydration of the lipid (in our lab we found that weighing which took less than 10 sec had no ill-effects for DLPC, DMPC or DPPC). Under all circumstances it is also a good practice to minimize the time the lipid is exposed to an oxygen containing atmosphere such as air as opposed to dry N₂ or Ar. Also, exposure of gramicidin and lipid to direct light for long periods of time should be avoided.
not totally anhydrous. Two methods for adding water to the sample mixture were tried in our lab.

Hydration of the sample mixture requires it to come into physical contact with water. Depending on the diffusion constant for the water into the sample mixture, the water will hydrate the sample mixture and after a period of time equilibrate with the mixture and the atmosphere. The first method to be mentioned involves exposure of the sample mixture to an inert atmosphere (N₂) in which the humidity has been controlled with salt solutions (Chemical Engineer's Handbook; O'Brien, 1948). The water uptake in phospholipid samples by humidity control has already been determined by other investigators (Levine et al., 1968, 1971). Close to maximum hydration (17-24% H₂O by weight) results after equilibration with a 95-100% relative humidity atmosphere for all phospholipids.

The desired humidity was maintained in the confined environment of a glove box filled with nitrogen gas by putting dishes of salt solutions in it. The sample mixture was removed from the vacuum and transferred to the glove box. It was placed on a watch glass and ground into a very fine and thin-layer in order that a large surface area would be exposed to the humidity atmosphere. Water absorption was inhomogeneous and equilibration at room temperature took on the order of two weeks; however, the rate for equilibration was increased for higher temperatures typically taking only 6-10 hours at 50-60°C.

The second method involved the direct addition of water to the sample mixture using a Hamilton syringe. In this case, after the sample was removed from vacuum, it was scrapped out of the 100ml round-bottom
flask and quickly reweighed. It was placed in a dental amalgamator capsule and the appropriate percent by weight of water was added with the syringe. The sample was lightly flushed with nitrogen and then tightly sealed in the capsule. It was mixed for approximately five minutes with the amalgamator and set aside so as to allow time to equilibrate. Water absorption was again inhomogeneous requiring 6-10 hours of incubation time at 50-60°C. In both methods, care had to be taken in keeping the sample environment free from contaminants such as dust. Therefore, the second method was preferred since it was easier to prevent contamination of the small amalgamator capsule compared to the large glove box.

We also required samples to be fully hydrated at the temperature they are to be kept (~24% by weight for pure DPPC samples just above 41°C). This was desired because the sample must be in the smectic A phase at low temperatures (<70°C). Samples were prepared and stored at low temperatures in order to prevent thermal denaturation of the protein. Also, defect structures such as oily streaks have more flow in fully hydrated samples\(^{10}\); thus, it was easier to swept them out of the sample under mechanical compression and dilation (Pershan and Prost, 1975; Asher and Pershan, 1979).

\(^{10}\) Maximum hydration refers to a sample which contains the most water by total sample weight but which is still in the smectic A phase. If the water content exceeds the maximum hydration limit then the sample enters into an undesired two phase region consisting of the lamellar phase and water. Although it is easy to swept defects out of maximum hydrated samples in the smectic A phase, it is almost impossible to remove defects out of samples in the two phase region.
Surface Treatment

Two methods for treating the glass substrates which helped enhance homeotropic alignment were used in our lab. The first method required treatment of the glass substrate with the surfactant N,N-dimethyl-N-octadecyl-3-aminoprophyltrimethoxysilyl chloride, DMOAP (Kahn, 1973). DMOAP is an alkoxy silane surface coupling agent which can bond to either glass or metal oxide surfaces. The alkoxy silanes will not bond directly with the surface but must first be hydrolyzed. The hydrolyzed saline molecules will then chemisorb on the hydrophilic surface forming a hydrogen bonded silane layer or an oxane bonded layer. The silane coating is cured by drying at room temperature or at an elevated temperature (<250°C) during which time the silane layer molecules are thought to polymerize to form a polysiloxane surface. Once polymerized the substrate surface becomes hydrophobic with a long hydrocarbon chain extended away from the surface as shown in figure IV-8. The hydrocarbon chains of the lipid molecules can now interdigitate between the hydrocarbon chains of the surfactant. This forms a monolayer of lipid molecules on top of the surfactant thus creating a boundary condition which allows water layers separated by lipid bilayers to stack up.

Before the surfactant can be properly deposited, the surface of the substrate must be thoroughly clean and smooth. Cleaning begins by dipping the substrate in benzene several times, swabbing with a Q-tip, and dipping again. The dipping and swabbing is then repeated using acetone instead of
benzene. The substrate is placed in a hot (~110°C) sulfuric chromic acid solution for approximately 5 minutes, removed and rinsed thoroughly with deionized/distilled water. The surface is clean if the final rinse totally wets the surface with no water beading. If the water beads then the substrate is dried and the hot acid mixture bath is repeated.

Once clean, the surfactant can be deposited. This is done by placing the glass substrate, still wet from the final cleaning rinse, in a dilute solution of DMOAP (typically 1% by volume in water), and agitating at room temperature for about 5 minutes. The substrate is rinsed in deionized/distilled water to remove excess silane; excess water is then blown free with a stream of clean dry N₂; and the silane coating is cured in dry N₂ at 110°C for ~1 hour. Substrates prepared in this manner are stable for weeks if stored in a dry nitrogen atmosphere.

The second surface preparation method involves scrupulous cleaning of the glass substrate with no surfactant treatment. In this case the surface remains hydrophilic. In view of the amphiphilic nature of the lipid molecules, it is not surprising that the hydrophilic surface would also help induce homeotropic alignment of the sample. In this method, the substrate is cleaned in the same manner as done for the surfactant treated substrate. However, the substrate is rinsed after removal from the hot acid solution with a distilled water steam bath; the condensing water rinses the surface. The hydrophilic surface is easily contaminated by organic deposits and dust even if it is stored in a nitrogen atmosphere; therefore, plates prepared in this manner must be used immediately after preparation.

Alignment of samples were obtained equally well with or without the
surfactant treatment. Usually the surface preparation was done immediately before the substrate would be used; thus, the majority of the substrates we used were not treated with the surfactant.

3. Alignment Techniques

The elastic free energy for non-planar configurations of a two-layer smectic liquid crystal with surface tension is higher than it is for the planar configuration when appropriate stabilizing boundaries are present. For example, if one or two glass plates are treated as described in the previous section and used as substrates, then they will impose the appropriate boundary conditions such that a system in the planar configuration will possess the lowest free energy. Hence, various techniques for alignment of smectic systems have been developed by liquid crystal researchers that exploit the energetics of the system and the applied boundary conditions. Descriptions of various techniques not used in our lab but commonly used by other researchers can be found in the literature and will only be briefly mentioned:

1) Evaporation techniques, developed by Libertini et al. (1969), result in thin dry or slightly hydrated single crystals of partially oriented phosphatidylcholine bilayers (300-3000 bilayers thick). Basically, this is accomplished by dissolving the desired lipid preparation in an appropriate solvent, placing a small amount of the preparation on a suitable substrate, and evaporating off the solvent. This process usually requires several days to complete during which time dirt and dust contamination may pose a problem. A variant of this method involves layer by layer deposition by
repeatedly dipping the substrate in a solution which contains the lipid and slowly retracting it under a stream of nitrogen to dry. The limited thicknesses produced by both processes do not allow a variety of experimentation, in particular limiting optical measurements.

2) The shearing technique was one of the earliest techniques used by liquid crystal researchers to produce partially aligned smectic systems (McFarland et al, 1971; Kleman et al, 1976). This technique takes advantage of the low resistance between shearing smectic layers. Samples of hydrated phosphatidylicholine are prepared by placing a small amount (5-15mg) between two clean and smooth substrates. After compression (squeezing) and/or shearing parallel to the substrate surfaces, partially aligned samples as thick as 20μ (~3000-5000 bilayers) are possible. Unfortunately, these samples are perturbed by many defects and only small (~1-3mm²) monodomain areas are typically produced.

3) An annealing technique by which thick bilayer arrays (~10⁵ bilayers thick) with large monodomain areas (~1cm²) was developed by Powers and Clark (1975). Orientated samples are obtained by placing lipid-water mixtures between two surfactant treated substrates clamped together with a teflon or glass spacer. The spacer seals and determines the thickness of the sample. The sample is heated to the high end of the temperature range of the smectic A phase a few degrees below the isotropic transition temperature. At this temperature range the sample's viscosity is a minimum and its diffusion constants and dislocation mobility are maximum. Hence, annealing of the distorted lamellar structure to its lowest free energy (planar) configuration is most rapid. The major
drawbacks of this technique are the high temperatures involved (~125°C for DPPC with 2% H₂O by weight), the long annealing time (typically 6-8 hours), and the low water content.

The alignment process used in our lab was pioneered by Asher and Pershan (1979). It is a combinational technique which employs mechanical stressing (compression/dilation) and low temperature annealing (<70°C). We chose this method because samples composed of hydrated lipid plus other biological components can be aligned at low temperatures in a few hours (~4 hours) which are 2-80μ thick (300-10000 bilayers) and have large monodomain areas (up to 40mm²). Although this technique does not produce monodomain areas as large as the high temperature annealing technique, the lower temperatures prevent possible protein thermal denaturation or lipid thermal decomposition. Samples with larger water content can also be directly prepared thus avoiding the cumbersome submersion method.

The mechanical compression and dilation presumably induces flow and stresses in the sample due to the changes in the sample thickness. The compression causes dislocations and other defects to flow out of the sample at stresses below the values that would create new defects (Pershan and Prost, 1975). While the defects are literally squeezed out of the sample, layers not coplanar with the glass surfaces can be broken. These broken layers heal and adopt the energetically favorable planar con-

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11 The water content can be increased up to the two phase region by submerging the already aligned sample in a temperature controlled water bath for a predetermined time (Powers et al. 1977).
figuration. Dirt and dust seem to aggregate in the "oily streak" defects and are swept out of the sample via these defects. The oily streaks are the most difficult defects to remove. Typically, the "oily streak" defect will not anneal away but must be mechanically pushed in one direction out of the center of the sample to an edge.

The low temperature annealing serves a two-fold purpose. Firstly, some defects such as the polygonal arrays, PFC, are created during mechanical dilation of the sample. Since the lipid has a negative thermal expansion, these types of defects anneal if the sample is slowly lowered in temperature. For example, a lowering rate of 0.5°C/min. from 60°C to 23°C works well for a fully hydrated DLPC sample with a gramicidin/lipid molar ratio anywhere from 1/50 to 1/300. Secondly, the sample must be above the gel transition temperature in the L\textsubscript{α} phase. The defect viscosity of the sample in the L\textsubscript{α} phase increases with increasing temperature. Therefore, defects are swept out or annealed away quicker at slightly higher temperatures in response to the mechanical stressing.

The alignment of the sample was as follows. A sample sandwich was made by clamping together two treated fused silica plates (1"x2"x1mm) with a spacer and the sample mixture in between. The spacer had a circular hole (~1cm diameter) punched in it and it was made out of mylar or silicone rubber. The spacer seals the sample and determines the sample thickness (2-80µm thick). Mylar was typically used for the thinner samples (<30µm). The compressibility of mylar is low; therefore, the bowing of the silica plates will induce most of the stress instead of the compression of the spacer as is the case for silicone rubber. The bowing of the silica plates
during compression caused enough stress for sufficient flow in the samples. Also, the mylar sealed the samples better than the silicone rubber preventing fewer air bubbles from entering into the sample or sample from leaking out.

Conoscopic interference or orthoscopic images between crossed polarizers were used to monitor the alignment process. A schematic of the conoscopic experimental setup is shown in figure III-2 and the orthoscopic experimental setup is shown in figure III-3. The experimental setups were flexible enough so that the sample could be interchanged between the conoscope and the polarizing microscope without excessively disturbing the equilibrium of the sample system. The sample holder was made out of stainless steel and is shown in figure IV-9. The two set screws press the top steel plate onto the sample sandwich thus securely sealing the sample in the hole in the spacer between the fused silica plates. Two heating resistors were mounted on the sample holder and the holder was fixed on the conoscope platform with the top of the glass cone touching the bottom glass plate. See figure III-2. The temperature of the sample was controlled by applying a fixed voltage to the two resistors in parallel. A calibration scale between voltage and temperature was predetermined and the voltage (temperature) was controlled via an APPLE-II/e computer and KEPCO BOP100-1M power supply with an IEEE-488 interface. The sample was slowly heated (~ .5°C/min.) to 60-68°C. This rate of increase was slow enough so that the sample remained close to thermal equilibrium. Once the sample reached the desired temperature, mechanical compression and dilation was achieved by one of two methods. First, the sample
sandwich, with the bottom glass plate in contact with the top of the glass cone, was lightly pressed and released on the glass cone. This caused the glass plates to bow enough to induce mechanical stresses in the sample. Sample flow was typically unidirectional causing defects to either anneal away or to be pushed out of the center of the sample area to one particular edge. Second, a system was rigged so that the top steel plate in the sample holder could be pressed and released on the top glass plate thus causing the sample to flow. Since the two set screws were fixed and already tightly adjusted, the second method was found not to be as effective as the first method.

Formation of polygonal arrays during dilation had little effect on the conoscopic interference patterns. If the polygonal arrays have formed then the patterns became slightly more diffuse. Observing this effect was not always easy. On the other hand, the polygonal arrays were easily seen using the polarizing microscope (with crossed polarizers) since the sample system is locally uniaxial with the symmetry axis normal to the lipid layers (the symmetry axis of the lipid layers is not perfectly parallel to the optical axis of the microscope thus the sample will be microscopically birefringent). Fortunately, most defects could be monitored using the conoscope. Once a sample appears perfectly aligned macroscopically under the conoscope, the temperature of the sample can be slowly lowered (~5°C/min.) to the temperature the sample is to be stored (i.e., for fully hydrated samples: room temperature for DLPC samples, ~24°C for DMPC samples, and ~42°C for DPPC samples). The sample can be viewed with the polarizing microscope so as to check for defects not revealed under the
conoscope. If the sample still has undesired defects then it can be warmed and the mechanical compression and dilation repeated.

Figures IV-10(b) shows a conoscoptic interference pattern of a homeotropic aligned fully hydrated DLPC sample with gramicidin/lipid molar ratio of 1/40. Figure IV-11 show the various stages of the alignment process as seen with the polarizing microscope with crossed polarizers. The dark areas are homeotropically aligned.
Fig. IV-1. Schematic representation of cross-sectional view of the molecular geometry of lipid liquid crystals in the $L_\infty$ or smectic A phase with gramicidin channels.
FIG. IV-2. (a) Phase diagram of the 1,2-dipalmitoyl-L-phosphatidylcholine-water (DPPC-water) system. (From Chapman et al., 1967). In the gel phase water separates the double layers of lipid, and the hydrocarbon chains are packed in an organized crystalline manner, probably in an hexagonal arrangement. The mesomorphic lamellar phase also has water separating the double layers of lipid; however, the hydrocarbon chains are now in a liquid state (melted). A cubic phase occurs at high temperatures and in the presence of only small amounts of water. It therefore occurs close to the left-hand side of the phase diagram. DLPC- and DMPC-water systems have similar phase diagrams as DPPC-water system but with lower values for $T_C$. (For DMPC, $T_C$=23°C; for DLPC, $T_C$=0°C). (b) A more detailed section of an optically deduced phase diagram for an oriented DPPC-water system, as obtained from a birefringence study. (From Powers and Pershan, 1977). Diagonal lines indicate two-phase regions and cross-hatching indicates regions where the phase diagram is not determined. In order for the annealing/mechanical method to work, the lipid-water system must be above the gel transition temperature in the $L_\gamma$ or $L_{\alpha'}$ phase. It is in this region that the lipid acquires enough flow so defects can be swept away. It is for this reason that preliminary experiments in our lab used DLPC with 20% water by weight since this lipid-water system would be in the smectic A phase at room temperature.
Fig. IV-3. Generation of the focal conic texture; (a) simple 'jelly-roll' or 'myelinic' arrangement generating a tube. (b) Tube closed into a torus: note the two singular curves (a circle and a straight line); (c) generalization: the circle becomes an ellipse, the straight line becomes a hyperbola and the smectic layers form Dupin cyclides. Figure was taken from DeGennes, P. G. (1974). "The Physics of Liquid Crystals". Clarendon Press, Oxford. p. 274.
Fig. iv-4. 'Oily Streak' defect or paired disclinations; (a) Established structure of defect in section perpendicular to the nominal disclination axes. The intersection of the edges of the molecular layers with the plane of the drawing is indicated by the solid lines. (b) Schematic diagram of the 'oily streak' defect shown in longitudinal section through the \( z = 0 \) plane. The cross-hatched areas mark the two central layers of each disclination. From Schneider, M. B., and Webb W. W. (1984). J. Physique 45, 273-281.
Fig. IV-4 (continued). (c) Optical properties of the structure in (a) and (b) as seen with a polarizing microscope with crossed polarizers. Polarizers are oriented as shown by the marks. Crossed hatching indicates areas with little light intensity. (d) Photomicrograph of an 'oily streak' defect in a fully hydrated DLPC multilayer sample with gramicidin A. Molar ratio is 1/40, thickness is 23.4μm, and temperature is 29°C. Schneider and Webb (1984) give a satisfactory account for the optical properties.
Fig. IV-5. (a) Three-dimensional structures of the locus of the cusps of the parabolic focal conics (PFC). (After Rosenblatt et al., 1977). (b) Schematic of the curvature of bilayers in PFC. (c) Boundary between the highly birefringent (brighter arrays) and PFC (dimmer arrays) polygonal arrays in a fully hydrated DLPC sample with gramicidin A. Molar ratio is 1/40, thickness is 23.4µm, and temperature is 65°C. The sample was in the process of being aligned. The microscope was focused at the center of the sample and the polarizers were oriented as shown by the marks.
Fig. iv-6. Strandlike defect in otherwise aligned domain caused by a fast temperature decrease (i.e., faster than $1^\circ$C/min). These defects usually run between other defects such as oily streaks, dust, air bubbles, etc. This photomicrograph of a strandlike defect was taken in the same sample as fig. iv-4,5; namely, a fully hydrated DLPC sample with gramicidin A. Molar ratio is 1/40, thickness is 23.4µm, and the temperature is 30°C.
Fig. iv-7. Highly birefringent globular areas are formed if compression/dilation are excessive during the alignment process. These areas appear to start from a singular point and slowly diffuse into circular "oily streak" defects under continued compression and dilation. The point to be made is: compression and dilation must be large enough so as to help anneal defects but small enough so as not to form defects. The sample was a fully hydrate DLPC sample (no gramicidin A). Thickness is 10μm, and temperature is 23°C. Double headed arrows depict the orientation of the crossed-polarizers.
N,N-dimethyl-N-octadecyl-3-aminopropyltrimethoxysilyl chloride (DMOAP)

\[
\begin{align*}
\text{C}_{18}\text{H}_{37} & \\
\text{Cl}^- + \text{N}-(\text{CH}_2)_3 \text{Si(OC}_3\text{H}_3)_3 \\
\text{CH}_3 & \quad \text{CH}_3
\end{align*}
\]

Fig. IV-8. DMOAP is a alkoxy silane. The above schematic shows the surface which results from the application of this surface coupling agent. (after Kahn, 1973)
Fig. iv-9. Cross-sectional view of the sample holder for the annealing/mechanical alignment technique. Heating resistors (not shown) would be mounted in parallel on both sides of the holder. Since the birefringence of the samples are typically very small ($\Delta n \sim .01$), a large light cone is needed in order to see the conoscopic interference patterns. If the light travels through a medium of high index of refraction (i.e., glass slide) into one of lower index of refraction then it is possible that the conoscopic interference patterns will be limited by the critical angle. Therefore, a glass cone that has a higher index of refraction than air is used so as to supply a medium in which the conoscopic interference patterns are seen. The glass cone, $n_{632.8}=1.521$, would be in contact with the bottom glass slide.
Fig. IV-10. (a) Conoscopic interference pattern of magnesium fluoride, MgF₂, crystal as seen with the conoscope. MgF₂ is a positive uniaxial crystal with a birefringence given by $\Delta n_{643.82\text{nm}} = .01176$ and $\Delta n_{587.56\text{nm}} = .01215$. The crystal is 840 $\mu$m thick. Note that the pattern consist of alternating dark circles and the illuminated circles that grade around in both directions from the 45-degree azimuths into a diffuse black cross. This is the characteristic pattern for a uniaxial crystal. Refer to "The Principles of Optics" by Born and Wolf for a full account on how this pattern arises. (b) Conoscopic interference pattern of a fully hydrated DLPC sample with gramicidin A. Molar ratio is 1/40, thickness is 23.4 $\mu$m, and temperature is 23°C. Since the birefringence is small and the sample is thin, the dark rings, as seen in (a), are outside the the light cone of the conoscope. However, the presence of the cross is enough to ensure that the sample is uniaxial and aligned. (The bright spot at the center of the cross is due to light reflection off of immersion oil.)
Fig. IV-10 (continued).
Fig. IV-11. The following photomicrographs were taken in time sequence during the annealing/mechanical alignment of a sample. The sample was a fully hydrated DLPC sample with gramicidin A. Molar ratio is 1/40 and thickness is 23.4μm. The spacer was made out of mylar. No surfactant was used. (a) A freshly prepared sample with no compression or dilation. Temperature was 23°C. The lines are mainly 'oily streak' defects separated by dirt. (b) The temperature was raised to 55°C at a rate of .5°C per minute. At this temperature, compression and dilation of the sample sandwich against the conoscope glass cone was done for about 5 minutes. The sample was then transferred to the polarizing microscope in order to take the picture. Already most of the dirt has aggregated into the nodes of 'oily streak' defects. (c) The temperature was further raised to 66°C. Compression and dilation was continued for another 15 minutes before taking the picture. The aligned areas are getting bigger and a few PFC are formed at the bottom of the picture due to dilation of the sample. (d) Temperature remained at 66°C and the compression and dilation was continued for another 30 minutes. Most of the dirt is gone and only 'oily streak' and PFC defects remain. (e) Compression and dilation of the sample was continued for another 30 minutes then the sample temperature was lowered to room temperature, 23°C, at a rate of .5°C per minute. The temperature decrease eliminated all of the PFC defects. A few strandlike and 'oily streak' defects can be seen in this photomicrograph. The strandlike defects usually anneal away in one or two weeks. Finally a sample that is 40mm² is aligned with over 97% of the sample defect-free.
V. Optical Rotatory Dispersion and Circular Dichroism

1. Introduction and Historical Perspective

Optical rotary dispersion (ORD) is a method of measuring the wavelength dependence of a molecule's ability to rotate the plane of linearly polarized light; circular dichroism (CD) is similar data evaluating the molecule's differential absorption of right- and left-handed circularly polarized light. The physical basis of ORD and CD is the same; therefore, both techniques reveal equivalent information about an optically active molecule based on how it interacts with polarized light. The nature of an optically active molecule (chromophore) arises from its asymmetric structure. However, although all the amino acids except glycine contain at least one asymmetric carbon atom, most amino acids display only small ORD or CD bands (Toniolo, 1970). The characteristic ORD and CD spectra of the proteins is due to the asymmetric and periodic arrangement of peptide units in space, in other words, due to the protein conformation. Since ORD and CD are very sensitive to changes in optical activity, protein conformational changes due to solvent environment, protein denaturation or helix-coil transitions are easily measured. Also, measurements of enzyme interactions with substrates, inhibitors, or coenzymes and of the binding of metal ions and dyes to proteins can be made.

The phenomenon of ORD had been known since the beginning of the nineteenth century. The pioneers included men such as Arago, Biot, Fresnel, and Pasteur. Table V-1 reports some important historical events related to
Table V-1 of Principal Historical Developments*

<table>
<thead>
<tr>
<th>Date</th>
<th>Authors</th>
<th>Concepts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1808</td>
<td>Malus</td>
<td>Polarization of light</td>
</tr>
<tr>
<td>1811</td>
<td>Arago</td>
<td>Changes of optical activity with wavelength (quartz)</td>
</tr>
<tr>
<td>1813-17</td>
<td>Biot</td>
<td>Optical activity of some organic substances</td>
</tr>
<tr>
<td>1824</td>
<td>Fresnel</td>
<td>Transverse vibration of polarized light</td>
</tr>
<tr>
<td>1846</td>
<td>Faraday</td>
<td>Magnetic optical activity</td>
</tr>
<tr>
<td>1847</td>
<td>Haedinger</td>
<td>Discovery of circular dichroism</td>
</tr>
<tr>
<td>1848-50</td>
<td>Pasteur</td>
<td>Optical resolution of racemic tartrates, optical isomerism</td>
</tr>
<tr>
<td>1896</td>
<td>Cotton</td>
<td>Circular dichroism in solution</td>
</tr>
<tr>
<td>1933-35</td>
<td>Mitchell,</td>
<td>First books on the Cotton effect</td>
</tr>
<tr>
<td></td>
<td>Lowry</td>
<td></td>
</tr>
<tr>
<td>1955</td>
<td>Djerassi</td>
<td>First paper of ORD and CD series by Wayne and Standford</td>
</tr>
<tr>
<td>1960</td>
<td>Grosjean,</td>
<td>Application of Pockels effect to CD instrument, First commercial dichrograph</td>
</tr>
<tr>
<td></td>
<td>Legrand</td>
<td></td>
</tr>
<tr>
<td>1969-71</td>
<td>Mazur,</td>
<td>First studies in linear dichroism</td>
</tr>
<tr>
<td></td>
<td>Yogev</td>
<td></td>
</tr>
</tbody>
</table>

* The references of these historical events can be found in Crabbe, P. (1972). "ORD and CD in Chemistry and Biochemistry". Academic Press, London.

the development of chiroptical methods (table taken from Crabbe, 1972) It was not until the turn of the twentieth century that ORD and closely related CD became physico-chemical tools in protein research. Of crucial importance in the development of these optical techniques were: 1) Staudinger's ideas that proteins are macromolecular substances (Staudinger, 1932), 2) the use of X-ray diffraction analysis in mapping of peptide backbone and side-chain positions of lysozyme (Phillips, 1966), several other enzymes, and quite a few other proteins in the solid state,
and 3) the results of analytical ultracentrifugation of protein solutions (Svedberg and Pedersen, 1940). These developments made it clear that protein structure should be accessible to ORD and CD. However, study of proteins by ORD was neglected until about 1955 because 1) the intricacy of the interactions between light and the macromolecules hampered the development of a theory which would permit a suitable interpretation of experimental data, 2) X-ray diffraction techniques dominated the field, and 3) there were limitations in ORD and CD instrumentation. The first commercial ORD spectropolarimeter became available in the mid-fifties making ORD measurements in the wavelength range above 240nm possible. By the mid-sixties, two revolutionary events caused a proliferation of polypeptide and protein investigation: 1) improved ORD spectropolarimeters including the spectral range 185-240nm and 2) the commercially available CD spectropolarimeters in the same spectral range. The increased spectral range now included the asymmetric peptide chromophore transitions. See Table V-2. The big advantages of CD over ORD for protein conformational studies are that 1) each optically active electronic transition gives rise to only one CD band instead of both positive and negative ORD signals (See fig. V-1), and the bands are thus more easily resolved and assigned, and that 2) CD, unlike ORD, bands are of finite width and, therefore, CD spectra contain no contribution from transitions outside of the measured spectral range (Adler et al, 1973). Therefore, CD became the dominate tool for optical activity measurements from about the mid-sixties to the present.

At present the theory of optical activity can not yield the precise structure of a protein from either ORD or CD spectra. Complications arise
### Table V-2: Chromophores, transition type and wavelength region

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Transition</th>
<th>Wavelength Region (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-C≡C-</td>
<td>π → π*</td>
<td>220 - 190</td>
</tr>
<tr>
<td>-C=C-</td>
<td>π → π*</td>
<td>200 - 185</td>
</tr>
<tr>
<td>-C=C-C=C-</td>
<td>π → π*</td>
<td>300 - 250</td>
</tr>
<tr>
<td>&gt;C=O, -CHO</td>
<td>π → π*</td>
<td>350 - 240</td>
</tr>
<tr>
<td>-C-C-C=O</td>
<td>π → π*</td>
<td>400 - 260</td>
</tr>
<tr>
<td>-COOH, lactone, ester</td>
<td>π → π*</td>
<td>280 - 200</td>
</tr>
<tr>
<td>Aromatic: Side chain</td>
<td>π → π*</td>
<td>300 - 250</td>
</tr>
<tr>
<td></td>
<td>π → π*</td>
<td>250 - 200</td>
</tr>
<tr>
<td>Skelton of terpene</td>
<td>π → π*</td>
<td>300 - 250</td>
</tr>
<tr>
<td>S-S</td>
<td>π → σ*</td>
<td>300 - 200</td>
</tr>
<tr>
<td>Protein, : Aromatic</td>
<td>π → π*</td>
<td>350 - 250</td>
</tr>
<tr>
<td>Poly-amide</td>
<td>π → π*</td>
<td>260 - 200</td>
</tr>
<tr>
<td>peptide transition</td>
<td>π → π*</td>
<td>260 - 185</td>
</tr>
<tr>
<td>DNA, RNA</td>
<td>π → π*</td>
<td>300 - 200</td>
</tr>
<tr>
<td>Co-chelate complex</td>
<td>d → d∞</td>
<td>700 - 300</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>300 - 185</td>
</tr>
</tbody>
</table>

From the asymmetrical perturbing effects caused by a chromophore's neighboring groups. Therefore, application of ORD or CD analysis to protein and polypeptide structure is usually empirical. One empirical approach obtains a standard ORD or CD spectrum for a particular protein whose structure is accurately known from techniques such as X-ray diffraction. This spectrum is then related to the structural features of the protein. An unknown protein spectrum can then be compared to this standard for analysis. The problem with this approach is that the structure of the protein in solution is assumed to be nearly the same as that used in the X-ray diffraction analysis (fiber, crystal, powder, etc. as prepared out of
the same solvent). Another similar empirical method was developed after the discovery of the water-soluble β conformation of heated poly-L-lysine. A standard ORD or CD spectra could be determined for each of the three major protein conformations, namely, α-helix, β form and random structures (Greenfield, 1967, 1969). See fig. 7-2. The ORD or CD spectra of an unknown protein can be compared to these standard spectrum in order to determine its conformational makeup. Problems with this method arise from the choice of the model system, effects of optically active nonpeptide chromophores, and light scattering effects. Also, it turns out that many proteins (gramicidins for example) have conformational structures other than the α-helix, β form or random coil.

Although ORD and CD analysis are empirical in nature, as mentioned earlier, great use can be made of the sensitivity of the methods to conformational changes. It is in this light that CD was applied to the gramicidin-lipid-water system. Since gramicidin A can adopt a variety of different conformations depending on its environment, it is desirable to known the CD spectra for the transmembrane channel conformation. Recall that experiments on gramicidin channels were primarily performed with two types of samples; namely, black lipid membrane and vesicular. It is only in the black lipid membranes that channel properties have been measured and only in the vesicular samples that CD spectra have be obtained. The gramicidin should have the same channel conformation in the aligned multilayers as in the black lipid membrane; thus, the CD spectra of these new samples will give the true transmembrane channel conformation CD spectra. Also, previously unmeasurable, these new samples will allow
the CD spectra to be measured during ion transport. Hence, any conformational change during this process can be measured.

Theoretical applications of ORD and CD can be found in the literature and only the classical theory is presented in the next section. Some good books which discuss various theories and their problems are listed in the reference section of this thesis (Velluz et al, 1965; Caldwell and Eyring, 1971; Charney, 1979; Mason, 1979; Harada, 1983; Jasco, Inc. J-500 A instruction manual, 1986; Frauenfelder, 1982).

2. ORD and CD Classical Theory

According to the theory of classical electromagnetism, when a beam of light passes through matter, the electric (E) field vector of the light interacts with the valence electrons of the component atoms. This interaction reduces the light velocity in the medium, that is, the refractive index n (=c/v) is not unity. This reduction in the velocity is known as refraction. Also, the medium decreases the amplitude of the E vector and is known as absorption. Since the light intensity is proportional to the square of the amplitude of the E vector, the initial intensity I₀ will be diminished in accordance with the following formula:

\[ I = I₀ e^{-kd} \] (a)

where I is the intensity of the light that has passed through the matter which is d cm thick, and k is the absorption coefficient (absorption). Refraction and absorption are, at the fundamental level, manifestations of
the same phenomenon; therefore, a correlation exist between the two expressions. In other words, if the absorption coefficient is known in the entire wavelength region, then it is possible, via the Kronig-Kramer formula, to determine the refractive index for any wavelength.

Linearly polarized light incident on an optically active medium will have the polarization plane of the transmitted light rotated. This can be understood by first noting that a combination of coherent right and left circularly polarized light of equal intensity produce linearly polarized light. The optically active medium interacts (refraction and absorption) differently with the light depending on whether it is right- or left-handed circularly polarized. Denoting the right- and left-handed polarization by the subscripts $r$ and $l$, respectively, the index of refraction and absorption coefficients are such that:

\[ n_r = n_l \]  \hspace{1cm} (b)  
\[ k_r = k_l \]  \hspace{1cm} (c).

Optical rotary dispersion is concerned with the difference in the index of refraction as given in equation (b). The measure of the angle of rotation of an optically active medium, expressed in degree units, is given by the following formula:

\[ \alpha_\lambda = (180d/\lambda) (n_l - n_r) \]  \hspace{1cm} (d)

where $\lambda$ is the wavelength of the incident light in vacuum and $d$ is the light pathlength. The specific rotation is defined by the following formula:
\[ [\alpha]_\lambda = \frac{\alpha_\lambda}{(dc)} \]  
\[ \text{(e)} \]

where \( d \) is the path length in dm, \( c \) is the concentration in g/ml, \( \lambda \) is the wavelength, \( t \) is the temperature, and \( \alpha \) is the angle the polarization plane has rotated in degrees. The molar rotation is commonly expressed by the formula:

\[ [M]_\lambda = [\alpha]_\lambda M/100 \]  
\[ \text{(f)} \]

where \( M \) is the molecular weight. The units of \([M]_\lambda\) are degrees·cm²·decimol⁻¹. If the substance under study is a polymer (polypeptide), it is more common to use the mean residual rotation to express the ORD:

\[ [m]_\lambda = [\alpha]_\lambda M_0/100 \]  
\[ \text{(g)} \]

where \( M_0 \) is the mean residue molecular weight (i.e., the molecular weight of the polymer divided by the number of monomers).

CD is concerned with the difference in the absorption as expressed by equation (c). The CD is usually expression as the absorption difference between the \( r \) and \( l \) light; that is,

\[ \epsilon_r - \epsilon_l = \Delta \epsilon \]  
\[ \text{(h)} \]

where \( \epsilon \) is defined by \( I = I_0 \cdot 10^{-\epsilon l c} \) and \( c \) is in mole/l. (Absorption coefficient \( k \) is nearly equal to 2.303\( \epsilon c \).) The differential absorption of the
right- and left-handed circularly polarized light means that the amplitude of the transmitted light will result in elliptically polarized light. The angle \( \theta \) whose tangent is equal to the ratio between the minor axis and the major axis of the elliptically polarized light is called the ellipticity, and the ellipticity expressed in degree units is related to the difference between both of the circular absorption coefficients:

\[
\theta = \left( \frac{180d}{4\pi} \right) (k_L - k_R)
\]  

(i)

Similar to the quantities defined for the optical rotation, specific ellipticity, molar ellipticity and mean residue ellipticity are used. First, the specific ellipticity is given by:

\[
\theta_\lambda = \theta / dc
\]  

(j)

and the molar ellipticity (or mean residue ellipticity) is written:

\[
[\theta]_\lambda = \theta_\lambda M / 100
\]  

(k)

where \( M \) refers to either the molecular or the mean residue molecular weight, \( d \) is the pathlength in dm, and \( c \) is the concentration in g/ml. The units of \([\theta]_\lambda \) are degrees cm\(^{-2}\) decimol\(^{-1}\). The molecular ellipticity can be expressed in term if \( \Delta \epsilon \),

\[
[\theta]_\lambda \approx 2.303 \left( \frac{4500}{\pi} \right) (\epsilon_L - \epsilon_R) \approx 3300 \Delta \epsilon
\]  

(l)

The relation between an ORD (\( \Delta n = n_L - n_R \) versus \( \lambda \)) and CD (\( \Delta \epsilon = \epsilon_L - \epsilon_R \))
versus \( \lambda \) spectra across an absorption band is shown in fig. 9-1. An ORD or CD curve of this type is called a Cotton effect. In case of the ORD, the Cotton effect is represented by the wavelength of the two extremes called the peak and trough, the amplitude, and the breadth. The amplitude is defined as

\[
a = ( [m]_1 - [m]_2 ) / 100 \quad (m)
\]

where \([m]_1\) is the extremum of molecular rotation on the longer wavelength side and \([m]_2\) is the extremum on the shorter wavelength side. A CD band is characterized by its magnitude at \(\lambda_0\) and breadth. The area under the CD band is known as the rotational strength, \(R_k\). It is express as

\[
R_k = \frac{3hc}{32 \pi^3 N_0} \int \frac{\Delta \varepsilon (\lambda)}{\lambda} \, d\lambda = 0.23 \times 10^{-38} \int \frac{\Delta \varepsilon (\lambda)}{\lambda} \, d\lambda \quad (n).
\]

The rotational strength describes the intensity of a CD band and includes physical information about the motion of the electrons when the chromophore is excited due to light absorption.

ORD and CD are related to each other by the Kronig-Kramers equation of integral transformation. The relations are written as

\[
[m]_\lambda = \frac{2}{\pi} \int [\theta]_\lambda \frac{\lambda'}{\lambda^2 - \lambda'^2} \, d\lambda' \quad (o)
\]

\[
[\theta]_\lambda = \frac{2}{\pi} \int [m]_\lambda \frac{\lambda'}{\lambda^2 - \lambda'^2} \, d\lambda' \quad (p).
\]
3. Circular Dichroism Measurement

CD spectra were measured with a Jasco J-500A spectropolarimeter. For vesicle samples, the CD measurement was taken at two positions, one 1" from the photodetector and the other 8", in order to check the effect of light scattering and absorption flattening inherent in particulate systems (Duysens, 1956; Urry, 1972b). Also, a short pathlength of 100μm was used for the vesicle samples so as to help reduce the possible light scattering problems. For all accepted spectra, the two measurements agreed within 5%. The CD spectra for gramicidin in vesicle suspensions are shown in fig. □-3. In a multilayer sample, the defect-free regions were transparent; therefore, light scattering was not a problem. Also, the appropriate background runs of either vesicle in water or multilayers (lipid+water) were subtracted from the measured spectra in order to obtain the CD spectra of the gramicidin. It should be mentioned that the multilayer CD spectra presented in fig. □-4 were taken from the defect-free region. However, the spectra practically remained the same when the detected areas were enlarged to include oily streak or polygonal arrays. No detection or evidence was shown that implied that gramicidin aggregates to the defects.
Fig. v-1. Relation between (a) an absorption spectrum ($\epsilon$ versus $\lambda$) and circular dichroism ($\Delta \epsilon$ versus $\lambda$) and (b) a dispersion curve ($n$ versus $\lambda$) and optical rotatory dispersion ($\Delta n$ versus $\lambda$) for an optically active substance. (c) The CD curve drawn as a solid line is called positive CD, and the dotted line is negative CD. (d) The ORD curve drawn as a solid line is called a positive Cotton effect, and the dotted line is a negative Cotton effect. From "Physical Biochemistry: Applications to Biochemistry and Molecular Biology" by Freifelder, D. M., 2nd ed., San Francisco, W. H. Freeman, 1982.
Fig. v-2. Circular dichroism spectra of poly-L-lysine in the 100% \( \alpha \)-helical (curve 1), \( \beta \) (curve 2), and random coil (curve 3) conformations. (From N. J. Greenfield and G. D. Fasman, Biochemistry 8, 4108-4116. (1969).)
Fig. v-3. CD spectra of gramicidin A incorporated in DLPC vesicles at three different protein/lipid molar ratios, R(G/L). The lipid concentration for each suspension was approximately 4mg/ml. The spectra at high molar ratio, R(G/L) > 1/50, have been reported in many publications (Urry et al., 1979; Wallace et al., 1981); they were considered the "standard" spectrum for gramicidin A in the transmembrane channel conformation. A mean molecular weight per residue of 124.5 was used to calculate the ellipticities.
Fig. v-4. CD spectra of gramicidin A incorporated in fully hydrated monodomain DLPC multilayers. At $R(G/L) \sim 1/40$, strong UV absorption made the spectrum below 225nm extremely noisy. (This was not a problem for the vesicle suspension since the UV absorption can be minimized by making the vesicle concentration low. The inset shows the shape of deformation of a membrane thicker than a gramicidin channel, G. A mean molecular weight per residue of 124.5 was used to calculate the ellipticities.
VI. Results and Discussion

The homeotropic alignment of lecithin multilayers was proven by the interference pattern produced by a conoscope, which indicated that the sample was uniaxial (Born and Wolfe, 1965). Our measurements of the birefringence (results not shown in this paper) of pure lecithin multilayers and multilayers containing gramicidin A agreed with the results of Powers and Clark (1975).

Previous CD studies of vesicle incorporated gramicidin A were mostly performed with samples with gramicidin/lipid molar ratios greater than 1/50 and a more or less "standard" spectrum was identified for gramicidin in the transmembrane channel state (Wallace et al., 1981; Urry et al., 1979). We reproduced this "standard" spectrum with a vesicle preparation which had a molar ratio of 1/40. See fig. V-3. However we found that at lower molar ratio values, e.g. 1/100, 1/200 and 1/300, the two main peaks, located at 217nm and 236 nm for molar ratios greater that 1/40, are progressively blue-shifted and broadened, so that at a molar ratio of ~1/300, the second peak becomes a shoulder of the first peak. A CD spectrum at low gramicidin concentration differing from the "standard" spectrum was noted previously by Ivanov and Sychev, 1982, but their spectrum was rather different from ours. They suggested the different spectra at low gramicidin concentrations was due to a different structure formed by association of three or more monomers. However, the spectra they reported appeared to be dominated by lipid effects rather than caused by a different gramicidin conformation. In contrast to such concentration
dependence, gramicidin incorporated in aligned multilayers shows a consistent CD spectrum for all molar ratio values from 1/40 to 1/300. We were not, however, able to obtain a reliable CD at \( \lambda < 220\text{nm} \) for 1/40 due to strong UV absorption even for a sample as thin as 2\( \mu \text{m} \); above 225nm the spectrum for 1/40 is identical to that of more dilute samples. In comparing the spectra in fig. \( \varphi \)-4 with the "standard" spectrum in fig. \( \varphi \)-3 the following was noted: 1) the spectra of multilayer samples are better defined because their peaks are narrower, 2) the peaks of the vesicle CD are blue-shifted relative to the multilayer CD by approximately 3nm, 3) the relative peak amplitudes are \([\theta]_{217}/[\theta]_{236} \sim 3\) for vesicles and \([\theta]_{220}/[\theta]_{239} \sim 2\) for multilayers, 4) all spectra for the multilayers have a negative ripple at \( \lambda \sim 280\text{nm} \), whereas the spectra for the vesicles go to zero. The differences described above are however exceedingly small if one compares these spectra with that of gramicidin in other solvent environments (Wallace, 1981). It can be concluded with good certainty that the spectra in figures \( \varphi \)-3 and 4 represent the same molecular conformation of gramicidin: fig. \( \varphi \)-3 for randomly oriented channels and fig. \( \varphi \)-4 for uniformly oriented channels. The small differences may be due to orientational effects (Cornell, 1979, 1982).

These oriented samples open the possibility of performing a variety of experiments, both electrical and spectroscopic in nature, on membrane incorporated proteins. Ion-conducting proteins other than gramicidin, such as melittin or alamethicin, can also be studied. Problems concerning protein structure, conformation, orientation and protein aggregation can be investigated in these new sample via dielectric relaxation, conduction,
small angle neutron scattering (SANS), extended X-ray absorption fine structure (EXAFS), and circular dichroism.

This paper showed that homeotropically aligned lecithin-water multilayers can be routinely prepared with or without other biological ingredients. Also, the circular dichroism spectra for gramicidin A in these samples revealed the polypeptide to be in the transmembrane channel state; therefore, new experimentation on gramicidin A in these new samples can be performed.
REFERENCES


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