Chapter 1

Determination of the Structure of Fluid Lipid Bilayer Membranes

Stephen H. White and Michael C. Wiener

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I. INTRODUCTION

The lipid bilayers of natural membranes generally exist in a fluid state which occurs above the gel-to-liquid-crystalline phase transition temperature. Knowledge of the structure of such “fluid” bilayers, which is of obvious importance for understanding the permeability and stability of membranes, can be obtained from X-ray and neutron diffraction measurements. However, fluid bilayers present special problems to the structural biologist. We are accustomed to viewing highly detailed images of protein crystallographic structures in which the mean relative positions of small groups of atoms are well defined and measurable. Diffraction studies of phospholipid crystals at low hydrations can provide a similar view of phospholipid molecules. Such three-dimensional images are not possible for fluid bilayers because of their inherent thermal motion and disorder. However, it is reasonable to consider the average translbilayer distribution of multiamom submolecular groups comprising the lipids and proteins. The “image” of the membrane in this case consists of the average spatial distribution of the submolecular groups projected onto a line normal to the plane of the membrane, from which the relative intergroup distances can be determined. It is this image, called a bilayer profile, which we take as “the structure” of the fluid bilayer. Even though it does not provide direct information about membrane structure in the other two dimensions (bilayer plane), this one-dimensional image lets us understand how lipid composition and proteins affect the translbilayer distributions of the submolecular groups.

We summarize in this chapter the methods we have developed for extracting quantitatively useful structural images of fluid bilayers from X-ray and neutron diffraction measurements by means of joint-refinement procedures. We first discuss the nature of the fluid-bilayer structure-determination problem and then the so-called composition-space refinement method for combining X-ray and neutron diffraction data. Finally, we discuss the fully resolved structural image of fluid bilayers formed from dioleoylphosphocholine.
II. THE NATURE OF THE FLUID BILAYER STRUCTURE PROBLEM

A. THE MEANING OF RESOLUTION

Most diffraction studies of fluid bilayers are performed using multilamellar arrays of bilayers formed from phospholipids by dispersal in water or deposition on glass substrates. The resulting lattice of thermally disordered bilayer unit cells typically yields five to ten orders of lamellar diffracted intensities from which the bilayer profile can be constructed.\textsuperscript{2,3,4} Such profiles have provided structural information such as the interbilayer headgroup separation\textsuperscript{4} and the presence of transbilayer acyl chain interdigitation.\textsuperscript{5} The profiles are expressed as transbilayer electron density, scattering-length density, or probability density depending upon the scale factors used with the Fourier transformation of the phased structure factors obtained from the diffracted intensities. Examples of X-ray and neutron scattering-length density profiles for bilayers formed from dioleoylphosphocholine (DOPC) bilayers are shown in Figure 1. Because the profile images are rather smooth and often lack fine structure, they leave the impression that they contain only modest amounts of information. In fact, they are rich in information because all of the atoms in the unit cell contribute to the profile. The structural problem is to decompose these profiles into subprofiles that describe the distributions of molecular fragments such as the phosphates, cholines, and carboxyl groups. Before the decomposition process can be appreciated, one must first understand the meaning of resolution.

The canonical resolution of the diffraction experiment is \( dh_{\text{res}} \), where \( d \) is the one-dimensional Bragg spacing and \( h_{\text{res}} \) is the highest order of diffraction observed. The Bragg spacing for bilayer systems is typically 50 Å with \( h_{\text{res}} = 5 \) to 10, so the canonical resolution is 5 to 10 Å. This resolution is often, and incorrectly, assumed to represent the limit on the accuracy with which the separation of structural features can be determined. In fact, the positions of resolvable features can be determined with a precision that greatly exceeds the canonical resolution.\textsuperscript{4} For instance, the high electron density peaks in the bilayer profiles obtained from X-ray diffraction (Figure 1B) are assigned to the phosphate moieties, and the distance between them (\( d_{\text{phosphate}} \)) is frequently cited to a precision of 1 Å or better.\textsuperscript{15,16,17,18} We refer to this aspect of resolution as resolution precision. The apparent conflict between the canonical resolution and the resolution precision can be resolved by a careful consideration of the nature of the disorder found in multilamellar fluid bilayer systems and its effects on the images obtained by Fourier transformation of the phased structure factors.

B. DISORDER, STRUCTURAL RESOLUTION, AND IMAGE RESOLUTION

One normally distinguishes three types of disorder in diffraction experiments.\textsuperscript{3,19,21} Disorder of the first kind is thermal disorder, in which the atoms or molecular fragments oscillate about well-defined positions within the unit cell. A sample with only this type of disorder will have a unit cell of well-defined composition and a lattice with a high degree of long-range order. Thus, even though the molecules of the unit cell are vibrating, the mean positions are well-defined and identical for all equivalent lattice positions. If thermal disorder is the only type of disorder present, then all of the diffraction peaks will be perfect images of the incident beam. That is, the widths of diffraction peaks will be independent of \( h \). The intensities of the peaks, however, will decrease more rapidly with increasing values of \( h \) compared to unit cells with less thermal disorder.

Disorder of the second kind, lattice disorder, applies to the situation where long-range order and/or uniform unit cell composition are lacking. For example, a pellet of biological membranes can consist of stacks of membranes of constant composition but with variable water spacing between membranes so that over long distances there is a loss of spatial coherence (i.e., a decrease in correlation length) between equivalent intrabilayer positions for pairs of membranes separated by many intervening membranes. When lattice disorder is present, the decreased long-range order causes the diffracted peaks to increase in width as \( h \) increases.

A third type of disorder is orientational disorder, which is related solely to the macroscopic features of a particular sample. For example, a single crystal of salt will produce discrete diffraction spots at well-defined angular positions relative to the X-ray beam. If the crystal is broken up to form a powder, the numerous small crystallites will be oriented at various angles with respect to one another and the X-ray beam so that ringlike diffraction spots are produced. The diffracting lattices can be nearly perfect in both cases.

The nature and quality of the image obtained in the diffraction experiment is governed by (1) the thermal disorder of the unit cell, which determines the maximum number of diffraction orders \( h_{\text{res}} \) that can be obtained under ideal conditions, (2) the number \( h_{\text{res}} \) of diffraction orders which are observable
Figure 1  Observed and calculated eight-order absolute scattering-length density profiles of DOPC bilayers (23°C, 66% RH). The units, scattering length per unit length, are dimensionless, and the density scale is multiplied by 10^4. The solid lines are the calculated profiles, and the curves with shading show the experimentally determined profiles including experimental error. A. Neutron density profiles. B. X-ray density profiles. The ordinate values for X-ray data, without the 10^4 factor, can be converted to electron density (e/Å^2) by dividing by 16.73. This factor includes the 10^4 factor, e^2/mc^2 to convert from scattering length to electrons, and the lipid area of 59.3 ± 0.7 Å^2 that was previously determined. (From Wiener, M. C. and White, S. H., Biophys. J., 61, 434, 1992. With permission.)

as result of the disorder of the lattice or other experimental conditions, and (3) the number of diffraction orders h_{j,r} actually used in the Fourier reconstruction of the image. Thermal disorder sets the ultimate upper limit on the image obtainable. For example, a well-ordered lamellar-like phospholipid crystal with a Bragg spacing of d ~ 50 Å whose atoms are confined to positions on the order of an atomic diameter (~ 2 Å) will produce about h_{max} = 50 Å + 2 Å = 25 orders of diffracted intensity as observed by Sakurai et al. and Suwalsky and Duk. The molecules of the unit cell in this case constitute a high-resolution structure because individual atoms or small groups of atoms can be resolved. With an excellent lattice and good experimental technique so that h_{min} = h_{max}, Fourier transformation with h_{j,r} = h_{max} yields a fully resolved image of the unit cell. However, if h_{j,r} < h_{max}, the resulting image
will be only a partially resolved image of the high-resolution structure. In contrast, a fluid bilayer structure is inherently a low-resolution structure because thermal motion causes the atoms of the molecules to be broadly distributed over distances of 5 to 10 Å so that \( h_{\text{max}} \) is limited to 5 to 10 diffraction orders in this case regardless of the carefulness of the sample preparation, the sensitivity of the detector, or the intensity of the source. In such a case, Fourier transformation using \( h_{\text{fp}} = h_{\text{max}} \) yields a fully resolved image of the low-resolution structure.

C. LATTICE ORDER AND RESOLUTION IN FLUID BILAYER SYSTEMS

Providing that lattice disorder or other experimental conditions do not limit \( h_{\text{max}} \), the above discussion makes clear that the difference in the number of diffraction orders observed from crystalline and liquid-crystalline phases is a direct consequence of the spatial distribution of matter resolvable over the time-course of a diffraction experiment. Individual atoms or small groups of atoms are discernible in the high-resolution structure of a crystal while the thermal disorder of the liquid-crystal causes these distributions to overlap, producing a low-resolution structure. The physically appropriate structural subunits of the liquid-crystal are these overlapping multiatomic “quasi-molecular” pieces.\(^8\)\(^,\)\(^9\)\(^,\)\(^13\)\(^,\)\(^24\) For both crystalline and liquid-crystalline materials, the intensities of the diffracted X-rays can be accurately measured, and in both cases models of appropriate resolution can be constructed which allow one to refine the structural image with great sensitivity. The average positions and widths of the distributions of multiatomic molecular “fragments” comprising the fluid bilayer system can be determined with considerable precision. This means that it is not correct to assume that the low canonical resolution of the bilayer diffraction experiment makes it impossible to determine distances and distributions to better than \( d/h_{\text{max}} \). On the contrary, if thermal motion is the only cause of disorder, then one can construct very accurate fully resolved images of the low-resolution structure.

The crucial question for the determination of fully resolved images of fluid bilayers is now clear: Can fluid bilayers form highly ordered lamellar lattices? In a series of elegant experiments utilizing synchrotron radiation and very high-resolution monochromators, Smith et al.\(^25\) demonstrated that arrays of \( L_\alpha \) phosphatidylcholine bilayers form virtually perfect one-dimensional lattices and that the widths of the observed Bragg peaks arise only from finite domain size. Most of their experiments were performed on highly oriented films at various hydration levels. This high degree of orientation, imperative for their detailed investigations of Bragg peak line-shape\(^25\) and bilayer in-plane structure,\(^6\)\(^,\)\(^27\) is not required for formation of highly ordered lattices. The observed line widths of Bragg peaks of oriented DMPC-cholesterol bilayers, prepared in a similar fashion to our samples, can be satisfactorily explained on the basis of purely instrumental effects.\(^4\)\(^,\)\(^28\) Specifically, the convolution of a Gaussian beam profile with an aperture representing the sample length in the beam adequately fits the measured line widths. This means the lattice is excellent with long-range order and uniform unit cell composition so that the diffraction is limited only by thermal motion, which reduces the intensity of higher-order spots. With this type of disorder, one is completely justified in representing the unit cell contents as a series of Gaussians whose widths describe the thermal motions of the represented pieces (vide infra).

It might at first seem peculiar that stacks of fluid bilayers with a great deal of thermal disorder produce a one-dimensional lattice with excellent long-range order. Though the phase space trajectory of the unit cell is very complicated, each unit cell is subject to the same constraints and interactions so that the average translayer distribution of matter is precisely the same for each bilayer. Because the bilayer can be considered a quasi-ergodic system,\(^20\) this distribution of matter representing the time-averaged dynamical structure of a single bilayer is equivalent to the spatial ensemble average of many bilayers’ configurations occurring at successive times, which gives rise to the observed diffraction. Thus, if one selects a particular point \( z \) within a particular bilayer, the average scattering density at that point will be precisely the same as the average scattering density at a point \( n \) bilayers away located at \( z + nd \). Fully resolved scattering density profiles of bilayers in a well-ordered lattice are therefore accurate representations of the average distribution of matter in the bilayers and can clearly reveal the thermal disorder, which is an important feature of the fluid bilayer. The determination of thermal disorder provides information on the relative dynamics of different portions of the molecule and is already a powerful technique in protein crystallography.\(^29\)
III. DETERMINATION OF FULLY RESOLVED IMAGES OF FLUID BILAYERS

A. JOINT REFINEMENT OF X-RAY AND NEUTRON DATA

The resolution of bilayer diffraction experiments can be functionally increased using neutron diffraction by taking advantage of the difference in neutron scattering length of hydrogen and deuterium.\textsuperscript{30-32} Specific labeling with deuterium at various positions within a lipid molecule makes it possible to locate the transbilayer position and distribution of the label with a precision of better than 1 Å.\textsuperscript{35-36} Such labeling has proven especially useful for determining the transbilayer location of added solute molecules such as hexane\textsuperscript{37} and peptides.\textsuperscript{8} The general difficulty with specific labeling experiments is the amount of chemical and diffraction work which must be done. For each labeled position, such as the acyl chain C(2) carbon of a phospholipid\textsuperscript{24} or the carbons of a double bond,\textsuperscript{11} one must typically repeat the diffraction experiment six times because the protonated and deuterated samples must be examined at several mole fractions of D\textsubscript{2}O in the aqueous phase to scale different data sets to one another, to reduce experimental uncertainty, and to determine the phasing. Heroic efforts are required to label enough different positions of the lipid molecule to arrive at a detailed image of the bilayer.\textsuperscript{34-36} To circumvent heroism, we developed a joint refinement method that combines X-ray data with minimum amounts of neutron data to arrive at detailed images of fluid bilayers.

The utility of considering both neutron and X-ray data in crystallographic structure analysis is well-established. The differences between neutron and X-ray structures of small-molecule crystals provide detailed information on the chemical bond.\textsuperscript{38,39} In protein crystallography, Schoenborn and co-workers\textsuperscript{40} elegantly combined neutron diffraction data with the X-ray structure of myoglobin to locate hydrogens and water in the structure. General procedures for the joint refinement of protein structures have been described by Wlodawer and Hendrickson.\textsuperscript{41} We extended these ideas to the refinement of bilayer structures.\textsuperscript{8} The joint refinement of the structure of a fluid bilayer by the combined use of neutron and X-ray diffraction data is based upon the significant differences in the neutron and X-ray scattering density profiles observed for phospholipid bilayers.\textsuperscript{29} Thus, the neutron scattering-length density profile is generally different from the X-ray scattering-length density profile because neutrons interact with nuclei whereas X-rays interact with electrons so that X-ray scattering is linearly related to atomic number whereas neutron scattering is not. Each experimental method thus "sees" a different representation of the molecule in its own scattering space, and each method has different sensitivities to various regions of the molecule: Neutrons scatter most strongly from the carbonyl groups of phospholipids because this part of the molecule lacks hydrogens whereas X-rays scatter most strongly from the electron-dense phosphate moiety.

B. QUASI-MOLECULAR MODELS: MULTI-GAUSSIAN REPRESENTATIONS OF BILAYERS

The principal objective of molecular modeling in bilayer diffraction studies should be to construct a real-space model for the distribution of matter across the bilayer which is both realistic and quantitatively useful. All modeling procedures involve the construction of a real-space model representing the transbilayer distribution of scattering length or electron density whose Fourier transformation will yield accurate estimates of observed structure factors in reciprocal space. A lipid bilayer can be equally well represented in reciprocal space by many different real-space models including strip models,\textsuperscript{26,42} "smoothed" strip models,\textsuperscript{29} "disordered" crystalline models,\textsuperscript{33,44-46} Gaussian models,\textsuperscript{24,35,47,48} and hybrid Gaussian/strip models.\textsuperscript{49} However, the various models are not equally useful in real space. The disadvantage of strip models is that the boundaries between different regions of the bilayer are discontinuous and thus unrealistic for a liquid-crystalline phase. This can be circumvented by appropriate smoothing with a Debye-Waller factor\textsuperscript{2,43} but one then encounters the problem of determining appropriate and meaningful Debye-Waller factors for the strips. More important, the compositions of the strips are generally unknown. The "disordered" crystalline model suffers from the fact that one must have the crystalline coordinates and, as with smoothed strip models, accurate knowledge of how to implement the disordering by means of Debye-Waller factors is required. Such knowledge is equivalent to knowing the molecular details of the transition from the crystalline to fluid state.

The Gaussian quasi-molecular model is a logical extension of the disordered crystal model in that Debye-Waller factors for small-molecule crystals are rigorously derived by considering the Gaussian-distributed deviations of atoms from their equilibrium positions.\textsuperscript{39} The one-dimensional projection of a perfect crystalline lipid structure along the bilayer normal is a series of sharp (approximately δ-function)
peaks. Thermal disorder, represented by the Debye-Waller factors of the constituent atoms, will broaden these peaks, leading to the "disordered" crystalline model. As thermal disorder increases, the broadened adjacent atomic peaks overlap, making it impossible to resolve them individually. It is logical to merge these overlapping and unresolved atomic distributions into a single Gaussian function representing an appropriate multiatomic grouping. The quasi-molecular model thus appropriately consists of a series of such Gaussians, which account for all of the atomic mass of the unit cell. The positions of the Gaussians represent the time-averaged positions of the submolecular pieces while their widths describe the range of thermal motion of the pieces. Because the quasi-molecular model accounts for thermal motion from the start, Debye-Waller terms are not included in the transform. The use of Gaussian distributions implies that the motions of these multiatomic distributions are primarily harmonic. In crystal structures, some atoms probably undergo anharmonic motion; however, molecular dynamics calculations suggest that these regions are best described by a series of Gaussians rather than a single non-Gaussian distribution.

The number of observable diffraction orders contains information about the fundamental structure of the bilayer and provides information on the number of Gaussians required to model it. The canonical resolution, $dh_{max}$, is the most appropriate length scale with which to describe the bilayer, i.e., it is the characteristic size of the molecular subunit that is discernible in the long-time (and space) average of a diffraction experiment. If an experiment records ten diffraction orders from a bilayer with a $d$-spacing of 50 Å, the principal scattering centers are approximately 5 Å wide. In the context of the quasi-molecular model, regions of the molecule that make the largest contributions to the total scattering are described by Gaussian distributions of 1/e-halfwidths of about 2.5 Å. Other regions of the molecule that contribute less to the total scattering may be more widely dispersed with larger widths, but the lower bound on distribution width is given approximately by the canonical resolution $dh_{max}$. Because of the importance of the canonical resolution in the determination of the appropriate length-scale, it is critical that all of the observable diffraction orders be measured. To determine the limits of spatial resolution, it is important to have a reasonable estimate of the experimental errors of the structure factors. A model based upon an imperfect data set, i.e., one that excludes significant higher-order structure factors, is likely to result in an incorrect model of the bilayer.

Through a series of simple model calculations, we demonstrated that analysis of membrane diffraction data can yield precise determinations of the centers of scattering density in liquid-crystalline bilayers. The physical basis underlying these calculations is the observation that multilamellar liquid-crystalline arrays, particularly single-component phospholipids and simple mixtures, are described by the convolution of a nearly perfect lattice with a highly disordered unit cell. As the width of a structural feature increases, the precision with which its center can be determined will decrease. The larger the scattering amplitude of a model component, the more precisely it will be located within the bilayer. This latter observation is completely consistent with the precise assignment of the major positive peaks in X-ray and neutron density profiles to phosphate and carbonyl moieties, respectively.

**C. COMPOSITION SPACE**

The thermal disorder makes possible a "composition-space" representation in which the quasi-molecular Gaussian distributions describe the number or probability of occupancy per unit length across the width of the bilayer of each component. This representation permits the joint refinement of neutron and X-ray lamellar diffraction data by means of a single quasi-molecular structure that is fit simultaneously to both diffraction data sets (Figure 2). Scaling of each component by the appropriate neutron or X-ray scattering length maps the composition space profile to the appropriate scattering length space for comparison to experimental data. Other extensive properties, such as mass, can also be obtained by an appropriate scaling of the refined composition space structure. Based upon simple bilayer models involving crystal and liquid-crystal structural information, we estimate that a fluid bilayer with $h_{max}$ observed diffraction orders will be accurately represented by a structure with approximately $h_{max}$ quasi-molecular components.

The fundamental composition-space joint-refinement method is based upon the obvious fact that, for thermally disordered liquid-crystalline bilayers, there is a single time and space-averaged bilayer structure that is invariant with respect to the type of beam used in the diffraction experiment. The quasi-molecular composition space model of a bilayer is a representation of this unique average real-space structure, which can be readily mapped to neutron and X-ray scattering spaces for comparison to experimental data. Because atomic neutron and X-ray scattering lengths have different physical origins,
Figure 2 Summary of the composition-space refinement method. The basic strategy is to determine probability or occupancy functions \( n(z) \) which describe the time-averaged transbilayer distribution of various parts of the hydrated lipid molecule. Scaling the functions by their neutron and X-ray scattering lengths should yield scattering-length profiles consistent with diffraction measurements. This approach recognizes the simple and obvious fact that there is a bilayer structure that is independent of the diffraction method used to determine it. Because X-ray and neutron scattering lengths are not related, the use of both diffraction methods effectively doubles the amount of data available for the construction of quasi-molecular models. (From Wiener, M. C. and White, S. H., Biophys. J., 59, 174, 1991. With permission.)

Each experimental method has different sensitivity to the constituent portions of the molecule. Further, because the two scattering lengths are unrelated, combining both data sets increases the available information for the structure determination. In essence, the diffraction data of one method serves to constrain or locate regions that the other method is less sensitive to. The resultant structure, surprisingly detailed, is more accurate than that obtainable from either neutron or X-ray data alone. In most cases, additional structural information must be obtained from deuterium labeling and difference-structure...
analysis in order to obtain the most detailed structure possible.\textsuperscript{31} However, one of the most significant advantages of the joint refinement method is the great reduction of the number of specific labeling experiments required to obtain a fully resolved image of the bilayer.

The time-averaged Gaussian probability distribution of each piece projected onto the bilayer normal can be described by

$$n_i(z) = \left( \frac{N_i}{A_i} \right) \frac{1}{\sqrt{\pi}} \exp \left[ - \frac{(z - Z_i)^2}{A_i} \right]$$  \hspace{1cm} (1)

where $n_i(z)$ is the fraction of the piece located at position $Z_i$ with $1/e$-halfwidth $A_i$ (Figure 2). The distribution can be viewed as the convolution of the hard-sphere or steric distribution of the quasi-molecular fragment with an envelope of thermal motion.\textsuperscript{10,11} In general, each piece $i$ consists of $N_i \geq 1$ identical subpieces. The $n_i(z)$ include the water molecules associated with the lipid and any other molecules contained within the unit cell. The precise meaning of equation (1) is important. For example, if the two carbonyl linkages (symbolized C=O) of phosphatidylcholine are represented by a single Gaussian function of unit area, then $n_i(z)$ is the fraction/unit length of the carbonyls at $z$ or the probability of finding the carbonyls' center-of-scattering at $z$. In particular, there are two carbonyls ($N_i = 2$) and $n_i(z)$ is the number of C=O groups per unit length at $z$ and $n_i(z) \div 2$ is the average occupancy per carbonyl at $z$ or the probability of finding a carbonyl at $z$. The distribution of matter across the bilayer can also be represented in terms of neutron scattering length or X-ray scattering length by multiplying Equation (1) by, respectively, the neutron scattering length $b_n$ or X-ray scattering length $b_x$ of piece $i$ so that the scattering length per unit length is

$$\rho^x_i(z) = b_j \cdot n_i(z)$$  \hspace{1cm} (2)

where $j = n$ or $x$. Thus, the neutron or X-ray scattering length per unit length at any point in the bilayer is given by

$$\rho^x_i(z) = \sum_{j=1}^{q} \rho^x_j(z)$$  \hspace{1cm} (3)

where $p$ is the number of quasi-molecular pieces per lipid. Because this equation is for a single hydrated lipid, it describes one monolayer of a bilayer extending from approximately $z = 0$ to $z = d/2$ or $z = -d/2$. We say approximately because near $z = 0$ and $z = \pm d/2$ portions of some pieces may spill over into neighboring half-unit cells. However, the portions “lost” will be recovered by spillover in the opposite direction from neighboring half-unit cells. For centrosymmetric bilayers, the amounts leaving and entering the half-unit cells are equal. The neutron and X-ray structure factors $F_i(h)$ of the model consisting of the set of $p$ quasi-molecular pieces are then given analytically by the Fourier transform of Equation (2) summed over all of the pieces:

$$F_i(h) = 2 \sum_{j=1}^{q} b_j N_i \cdot \exp \left[ - \frac{(\pi A_i h)^2}{d^2} \right] \cos \left( \frac{2\pi Z_i h}{d} \right)$$  \hspace{1cm} (4)

The rule of thumb for the number of quasi-molecular components is that the number of Gaussians $p$ is approximately equal to the number of observed lamellar diffraction orders.\textsuperscript{9} While $p \sim h_{max}$ is the number of Gaussians required to obtain a complete and accurate structure of the entire bilayer, specific regions of the bilayer, particularly those that are major contributors to the total scattering, can be readily located in simpler models with fewer Gaussians if a complete solution is not required. There are many ways to divide a lipid molecule into $p$ fragments, but two important guidelines simplify the process. The first guideline is to parse the hydrated molecule into $p \sim h_{max}$ pieces that have widths $2A_i \sim d/h_{max}$. The second guideline is inherent in the composition space refinement method. Namely, the positions $Z_i$ of the pieces must be the same in both X-ray and neutron scattering-length spaces. This entails parsing the atoms among the pieces so that the weighting by the scattering lengths, Equation

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leads to model scattering-length profiles consistent with the observed ones. The appropriate parsing is ultimately determined by experimental sensitivity and the relative widths and scattering lengths of the distributions. The parsing must be done largely by trial and error in specific cases.

**D. MODEL REFINEMENT**

The structural model is determined by finding the set of composition-space models that yield the best agreement to both the neutron and X-ray data. Nonlinear minimization with the standard Levenberg-Marquardt algorithm is carried out to determine the parameters \( Z \) and \( A \) of Equation (4), which minimizes the joint crystallographic \( R \)-factor defined here as

\[
R = \sum_{F_{\text{obs}}} R_j
\]

where

\[
R_j = \frac{\sum |F_j(h) - |F_j^* (h)||}{\sum |F_j^* (h)|}
\]

\( F_j^* (h) \) are the experimental structure factors scaled to the appropriate relative absolute scale. A composition-space structure is judged to be satisfactory if it provides fits to both the neutron and X-ray data sets that are below the experimental noise or "self-R". The robustness of the structure determination and the uncertainties in the parameters are examined by introducing Gaussian-distributed noise into the data sets. Each of the absolute neutron and X-ray structure factors has an associated uncertainty, which is used to define the width of a normal distribution centered at the best value of the structure factor. Monte Carlo methods are used to select mock data from these distributions, which are used as the input for the structural calculations.

**IV. THE STRUCTURE OF A FLUID BILAYER**

We obtained the complete structure of 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) in the \( L_n \) phase (66% RH, 23°C) by the joint refinement of neutron and X-ray lamellar diffraction data. The requirement that a successful quasi-molecular model fit two independent sets of data strongly constrains the ways in which the molecule can be divided. Figure 3 depicts the quasi-molecular model of DOPC and its associated water molecules that was used in the structural determination. This one was chosen initially because it logically identified the obvious molecular fragments. We subsequently examined more than 30 other parsing schemes but none of them led to successful refinements. The methylene region (part number 2) in Figure 3 is represented by three Gaussians, so ten quasi-molecular fragments were required to obtain the complete structure of the DOPC bilayer. Each piece requires three parameters: position \( Z \), 1/\( e \)-halfwidth \( A \), and area \( N \). The water and double-bond distributions were determined independently from neutron diffraction experiments, which reduced the number of parameters from 30 to 24. The terminal methyl distribution was determined from a direct combination of neutron and X-ray data prior to the full joint-refinement, so the parameter set was further reduced to 21. The contents of each of the remaining pieces of the model, except for the methylene envelope, were fixed by the parsing so that only the positions and 1/\( e \)-halfwidths were determined during the nonlinear minimization. Specifically, the contents of the carbonyl, glycerol, phosphate, and choline fragments were fixed so that the number of parameters was reduced to 17. We estimated previously that a fluid bilayer yielding \( h_{\text{max}} \) diffraction orders would require \( p \approx h_{\text{max}} \) quasi-molecular Gaussian distributions to describe it adequately. We had \( h_{\text{max}} = 8 \) orders for both X-rays and neutrons and \( p = 10 \) Gaussian fragments consistent with the approximation.

**A. GENERAL FEATURES OF THE STRUCTURE OF DOPC BILAYERS**

The complete structure of DOPC bilayers at 66% RH is shown in Figures 4 and 5. Figure 4A depicts the structure excluding the headgroup (phosphate and choline) and water distributions and Figure 4B
the headgroup and water distributions and the overlap with the methylene distribution. Figure 5 shows two bilayer leaflets head-to-head, with the water, phosphate, choline, glycerol, and carbonyl fragments indicated. Figure 6 shows the real-space of the 50 methylene profiles for a single lipid determined during the refinement; the conservation of the shape of the total methylene envelope demonstrates the robustness of the result. Although the methylene region contributes a major fraction of the total X-ray scattering, it is spread out over a wide range, so its effective contribution is reduced. While the contribution of a quasi-molecular fragment to a structure factor is proportional to its total scattering length, the width of the distribution appears as an exponential factor that dominates the structure factor amplitude [Equation (4)].

The structure of liquid-crystalline L_{α}-phase DOPC at 66% RH (5.4 waters/lipid, Reference 55) shown in Figures 4 through 6 is the complete and fully resolved image of the bilayer as seen over the timescale of the diffraction experiment. The combination of the two independent X-ray and neutron data sets precludes the necessity of copious specific deuterium that was previously required for detailed determination of structural features by neutron diffraction.34-36 The image of the bilayer obtained is consistent with the wealth of information gathered over the past decades by spectroscopic and structural methods. Importantly, however, the structural image obtained here is based entirely upon the absolute neutron and X-ray structure factors.

An approximate average tilt-angle of the phosphocholine dipole with respect to the bilayer surface can be estimated from the distance between the centers of the phosphate and choline pieces along the bilayer normal. Assuming that the phosphorus and nitrogen atoms are the centers of scattering of each of these roughly spherical fragments and a phosphate-nitrogen distance of 4.5 Å is obtained from the crystal structure of DMPC,4 the dipole is calculated to be canted with an angle of 22 ± 4° with respect to the bilayer surface. This compares favorably with the values obtained from crystal structures and neutron diffraction of oriented multilayers33 and is in reasonable agreement with the recent value of 15° obtained from 'H-NMR and Raman spectroscopic studies of L_{α}-phase DPPC.35

In bilayers at reduced hydration, steric factors are believed to play a major role in interbilayer interactions.37 Figure 5 shows clear evidence of steric effects between apposed bilayers at the moderate hydration of our experiments. There is significant overlap between the choline distributions and, to a lesser extent, between the phosphate distributions of the two “head-to-head” bilayer leaflets. In projection, there are regions of space that are accessible to both headgroups, i.e., the position of the headgroup of one bilayer can affect or exclude the other’s position. A question of obvious interest is how this steric

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- **Figure 3** The parsing of DOPC into the quasi-molecular parts used in the structure determination by the joint refinement of X-ray and neutron data. (From Wiener, M. C. and White, S. H., Biophys. J., 61, 434, 1992. With permission.)
Figure 4. Composition-space structure of DOPC. A. The pieces corresponding to the bilayer interior: methyls, methylenes, double bonds, carbonyls, and glycerol backbone. B. The headgroup region: phosphate, choline, and water. The methylene region is also indicated. (From Wiener, M. C. and White, S. H., Biophys. J., 61, 434, 1992. With permission.)

interaction, clearly seen as an overlap of apposed headgroups in Figure 5, will change as the hydration level increases. Determining fully resolved structures over a range of hydration will provide valuable information on this issue and others pertaining to the hydration force.19,38

B. THERMAL MOTION

Because the real-space image is physically meaningful, there is useful and interesting information in the widths of the Gaussian distributions that characterize each quasi-molecular fragment. The positions of the distributions denote the most likely place to locate the center of scattering of each fragment, whereas the widths describe the range of thermal motions projected onto the bilayer normal assuming undulatory motions are insignificant in our system. The 1/e-halfwidth A, of a quasi-molecular Gaussian fragment can be viewed as the convolution of a “hard sphere” of van der Waals radius \( D_v \) located at \( Z \), with a Gaussian envelope of thermal motion describing the range over which that piece moves within
Figure 5 Composition-space structure of two apposed DOPC leaflets. The overlap of the cholines, and to a lesser extent of the phosphates, is clearly indicated. Water is seen to penetrate the bilayer to the extent of the glycerol backbone fragment. (From Wiener, M. C. and White, S. H., Biophys. J., 61, 434, 1992. With permission.)

Figure 6 Composition-space averaged methylene envelope. Although the three-Gaussian basis set that comprises the methylene distribution is degenerate, the resultant sums of the three Gaussians are conserved. (From Wiener, M. C. and White, S. H., Biophys. J., 61, 434, 1992. With permission.)

the bilayer. The observed 1/e-halfwidth is given approximately by \( \sqrt{D_R^2 + D_I^2} \), where \( D_I \) describes the envelope of thermal motion. Because of the approximate nature of this crude expression and the ambiguity in estimating hard-sphere widths of each of the quasi-molecular fragments, we did not explicitly repeat the calculation for all of the fragments. The narrowest thermal distribution is that of the glycerol region (\( \lambda_{\text{avg}} = 2.46 \pm 0.38 \) Å). The 1/e-halfwidths of the quasi-molecular pieces on either side of the glycerol backbone increase as shown graphically in Figure 4A. The general image is a “gradient” of thermal motion within the interface zone in which the regions bounding the relatively rigid glycerol backbone undergo increasing ranges of motion that are roughly proportional to the distance from the glycerol “fulcrum.” This is consistent with NMR results\(^\text{50,60} \) and crystallographic
measurements,\textsuperscript{44,45} which indicate that the glycerol backbone is the most rigid portion of the liquid-crystalline phospholipid bilayer on DMPC. It is interesting in this context that the glycerol region is at the extreme boundaries of both the methylene (Figure 4A) and water distributions (Figure 5) and thus marks the water-methylene interface. The net thermal motions within the hydrocarbon region, compared to the interface zone, are qualitatively different in that the 1/ε-halfwidth of the terminal methyl groups (2.95 Å) is about the same as the carbonyl (2.77 Å) or phosphate groups (3.09 Å), while the width of the double-bond distribution is significantly larger (4.29 Å). This apparent violation of the notion of a gradient of thermal motion may be explained if one treats the flexible acyl chain as being “tethered” at one end to the interface by the carbonyls and at other end to the bilayer center by the terminal methyls. Because the half-thickness of the hydrocarbon is considerably shorter than the length of the fully extended chain, the tethering would permit the double bonds to diffuse over a relatively large volume of space. It would also permit some of the methylene to venture beyond the C(2) carbons into the interfacial zone (vide infra).

C. VOLUMETRIC MEASURES OF BILAYER THICKNESS

Useful bilayer thickness information has been obtained in the past by using simple equations which use density and volume data to calculate the thickness of a uniform “slab” of bilayer material. Given \( S_0 \) (the area/lipid), the equivalent-slab thickness of a component(s) of the bilayer of molecular volume \( V \) is \( V/S_0 \). The several methods of this type which are in common use make various assumptions about which parts of the lipid molecule are included in \( V \) for the calculation of a bilayer thickness. Smail\textsuperscript{82} excluded the phosphocholine group to obtain a “lipid thickness” \( d_L \) of egg lecithin bilayers, assumed to be equivalent to a slab of 2 diacglycerols. Luzzati and his co-workers\textsuperscript{83-84} included the entire phospholipid molecule to arrive at a “bilayer thickness” \( d_B \). Nagle and Wiener\textsuperscript{45} extended these “slab” models to include phospholipid bilayers under a variety of physical conditions to obtain, among other parameters, the idealized hydrocarbon thickness \( 2D_C \), which is calculated from the volumes of the acyl chains excluding the carbonyl groups. If the average widths of regions of the bilayer obtained from simple volumetric formulas correspond to positions of quasi-molecular fragments obtained from joint refinement, then these volumetric methods could be utilized in future studies to constrain further the joint refinement procedure. These calculated thicknesses are compared with the model structure in Figure 7, which shows that \( d_L/2 \) and \( d_B/2 \) correspond within experimental error to the positions of the glycerol and choline peaks, respectively. Interestingly, the mean of \( d_L/2 \) and \( d_B/2 \) corresponds closely to the position of the phosphate group.

D. THE HEADGROUP-HYDROCARBON BOUNDARY

In the absence of other structural information, the boundary between hydrocarbon and headgroups has generally been taken as the edge of the equivalent slab comprised of the acyl chains between the C(2) carbons,\textsuperscript{46-49} so that the hydrocarbon thickness \( 2D_C \) calculated in the manner of Nagle and Wiener\textsuperscript{45} was assumed to mark the positions of the boundary. The equivalent hydrocarbon slab of thickness \( 2D_C \) has been superimposed on the quasi-molecular model in Figure 7B, and it can be seen that the slab edges correspond rather precisely to the positions of the carbonyl groups. Assuming the volumes of the methylenes, double bonds, and methyls to be 27, 43, and 54 Å\textsuperscript{3}, respectively, \( D_C \) is found to be \( 16.0 \pm 0.2 \) Å compared to \( Z_{COO} = 15.99 \pm 0.06 \) Å.

The mean position of the carbonyls is the most accurately determined position of the quasi-molecular model because it is the most strongly scattering feature in neutron diffraction experiments. The mean position of the C(2) carbons is constrained by the covalent carbonyl-C(2) bond not to exceed 1.54 Å; although the precise separation between the carbonyl and C(2) is thus uncertain, it is reasonable to assume that the average position of the C(2) is on the hydrocarbon side of the carbonyl rather than the phosphorylcholine side. This suggests that the average direction of the axis through the \(-\text{O}=-\text{CO}-\text{CH}_3\) segments will tend to be parallel to the bilayer plane. This general arrangement has been observed in oriented L\textsubscript{a}-phase dimyristoyl- and dipalmitoylphosphatidylcholine multilayers by Hübner and Mantsch\textsuperscript{90} by means of FT-IR spectroscopy. Those authors also reported, however, that the orientations of the \(-\text{O}=-\text{CO}-\text{H}_3\) segments at the sn-1 and sn-2 positions were different, but our structure provides no information on this point.

Although the positions of the carbonyls mark the hypothetical hydrocarbon slab boundaries, such boundaries belie the chaotic nature of the interface and, in reality, do not apparently include all of the methylenes. The average number of methylenes extending beyond the slab edges is equal to the area
between the methylene envelope and slab boundary in Figure 7B, which is 3.7, or about two methylene groups per hydrocarbon chain. Interestingly, this number is about equal to the average number of methylenes one expects to be exposed to water when the area per lipid increases from 40 Å² in the crystalline all-trans state to 60 Å² in the Lα phase. Aveyard and Haydon\textsuperscript{71} estimated the cross-sectional area of a single methylene group projected onto a plane interface to be 6 Å²; so an excess area of 20 Å² corresponds to about 3.3 methylenes. Because the average position of the C(2) carbons is displaced from the carbonyl in the direction of the bilayer center,\textsuperscript{24} these methylenes cannot be attributed entirely to the C(2) carbons and must therefore generally be from more distant carbon positions. This observation
would seem to place definite constraints on the allowable chain conformations and is consistent with the idea that the chains may behave as though they are tethered at each end.

**E. BILAYER THICKNESS DYNAMICS AND PERMEABILITY**

We believe that the most important observation of this work is the extent of the transbilayer thermal motion of the quasi-molecular fragments, which must be taken as a fundamental property of fluid bilayers. There is significant overlap between the distributions that comprise the bilayer so that various regions of the bilayer, such as the headgroup/water interface, consist of a dynamic mixture of components with very different physical and chemical properties. The static image of the bilayer implicit in slab representations can now be replaced with a realistic dynamic image. This image allows one to think more clearly about the problem of how molecules in the aqueous phase can penetrate the bilayer. Because of the thermal motion, there can be a higher probability of polar molecules such as water penetrating, at least transiently, deeper into the hydrocarbon core than expected on the basis of slab models. This means that the thickness of the bilayer is dynamic with respect to the transbilayer separation of water. This situation is shown in Figure 8, where we have plotted the distributions of the methylenes, glycerols, double bonds, and water. An important feature of this plot is the small but significant overlap of the water and double-bond distributions indicated by the vertical lines. This overlap suggests to us that there must be transient contacts between the double bonds and water, which may play a role in water permeation of the bilayer. Petersen has inferred from permeability studies of black lipid films that a single unsaturated bond dramatically increases the solubility of water suggesting an association of water with double bonds. Noting in Figure 8 that there is a small overlap of the double bonds at the bilayer center, we can speculate that the double bonds might ferry water across the bilayer.
If one takes the maximum extent of water permeation into the bilayer as a measure of the minimum transient thickness (which we shall call the dynamic thickness) of the bilayer, the large arrow in Figure 8 shows that the effective thickness of the hydrocarbon core can be less than 28.6 Å compared to the 32 Å slab thickness where 28.6 Å is determined by the "crossover" points of the double-bond and water distributions. Although a 3 to 4 Å difference is not large, it is important to remember that the hydration of our lipids is relatively low. As the hydration is increased, the mean bilayer thickness will decrease and the thermal motion will increase. For DOPC in excess water, $S_0$ increases to 70 Å$^2$ (Reference 73) causing $2D_c$ to decrease to 27 Å. We have been able to observe no more than 3 diffraction orders from DOPC under these conditions. Using our rule of thumb that the typical full width of a quasi-molecular fragment is \( dl/h_{tot} \) (Reference 8), we can expect the width of the distribution of the double bond to increase from about 8 Å to 12 Å or more depending upon the amount of simple thermal motion relative to the undulatory motion which may be present.\(^{15,25} \) We speculate that there will be a concomitant increase in the width of the water distribution leading to a dynamic thickness considerably smaller than the static one. It would thus appear that for dynamic processes, the effective thickness of bilayers may be much smaller than slab models would indicate. We speculate that as the method is extended to phospholipids at high hydration, very significant increases in transbilayer thermal motion and equally significant decreases in the dynamic thickness of bilayers will be observed. If this speculation proves to be true, it may become easier to understand how seemingly difficult processes such as the transport of proteins into and across membranes can occur.

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**REFERENCES**


