

Fluid bilayer structure determination by the combined use of x-ray and neutron diffraction

II. "Composition-space" refinement method

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ABSTRACT This is the second of two papers describing a method for the joint refinement of the structure of fluid bilayers using x-ray and neutron diffraction data. We showed in the first paper (Wiener, M. C., and S. H. White. 1990. *Biophys. J.* 59:162–173) that fluid bilayers generally consist of a nearly perfect lattice of thermally disordered unit cells and that the canonical resolution d/h_{\max} is a measure of the widths of quasimolecular components represented by simple Gaussian functions. The thermal disorder makes possible a "composition space" representation in which the quasimolecular 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 quasimolecular structure that is fit simultaneously to both diffraction data sets. 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 $\sim h_{\max}$ quasimolecular components. Strategies for assignment of quasimolecular components are demonstrated through detailed parsing of a phospholipid molecule based upon the one-dimensional projection of the crystal structure of dimyristoylphosphatidylcholine. Finally, we discuss in detail the number of experimental variables required for the composition space joint refinement. We find fluid bilayer structures to be marginally determined by the experimental data. The analysis of errors, which takes on particular importance under these circumstances, is also discussed.

INTRODUCTION

We continue in this paper the presentation of the principles of analyzing fluid bilayer diffraction data to arrive at accurate quasimolecular structures. The previous paper established the utility of quasimolecular models in the interpretation of lamellar diffraction data from liquid-crystalline bilayers and investigated their limits of resolution (Wiener and White, 1990). A satisfactory quasimolecular model was shown to consist of a collection of Gaussian distributions, each representing a submolecular fragment of the hydrated lipid molecule. We demonstrated that the approximate widths of quasimolecular components are given by d/h_{\max} , where h_{\max} is the highest diffraction order observed. Moreover, we showed that the centers of these broad thermally disordered distributions can be located with a precision of 0.1–0.5 Å from typical data sets. In the present paper, we (a) introduce the "composition space" method for the joint refinement of neutron and x-ray data, (b) develop guidelines for selecting an appropriate quasimolecular model, (c) discuss the experimental degrees of freedom of the method, and (d) discuss the analysis of errors.

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 crystals provide detailed information on the chemical bond (Coppens, 1967, 1974). In protein crystallography, Schoenborn and co-workers (Norvell et al., 1975) 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 (1982) but, as far as we can establish, no general procedures have been developed for the joint refinement of bilayer structures. In some limited applications, McCaughn and Krimm (1982) considered the combined use of neutron and x-ray data to extract information about the composition of bilayers in well-defined regions, and Herbet et al. (1985) used water and lipid profiles obtained from neutron diffraction in the analysis of x-ray diffraction data from sarcoplasmic reticulum membranes. There are two advantages to combining neutron and x-ray data. First, 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

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not. This makes it possible to extract additional useful structural information by combining the two methods. Second, because neutron beams have small fluxes and because experimental time on neutron sources is limited, it is advantageous to take maximum advantage of the ready availability of x-ray beams.

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 quasimolecular 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, each experimental method has different sensitivity to the constituent portions of the molecule (Franks and Lieb, 1981). 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 must be used (Büldt et al., 1979) to obtain the most detailed structure possible. 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.

We showed in the first paper of this series that the number of observable structure factors depends strongly on the $1/e$ half-width of the quasimolecular distribution. As the half-width increases, the number of observable structure factors decreases sharply. For typical liquid crystals, 5–10 diffraction orders are observed. We present here calculations which demonstrate that the number of observed diffraction orders indicates the number of quasimolecular components required to describe the bilayer completely. In addition, we use the crystal structure determined by Pearson and Pascher (1979) for dimyristoylphosphatidylcholine (DMPC) as a basis for a hypothetical quasimolecular model to demonstrate in a more detailed and realistic way the fundamental features of quasimolecular modeling and joint refinement analysis. Differences between structures based solely upon either neutron or x-ray data are clearly shown. Although there are a myriad of ways in which one can subdivide a lipid molecule and any other components of

the membrane into quasimolecular fragments, we conclude that a reasonable initial guess at parsing can be made from consideration of the chemical structure and existing physicochemical data. Refinement of the parsing is carried out in the course of determining the composition space profile (or set of profiles) that adequately fits both data sets. A meaningful refinement requires that the model be adequately determined experimentally and we therefore discuss the experimental degrees of freedom of the problem. We show that the problem is marginally determined; this places special emphasis on error analysis which we also discuss.

"COMPOSITION SPACE" REFINEMENT METHOD

Consider a one-dimensional lattice of hydrated liquid-crystalline bilayers of repeat period d . Let the hydrated lipid be subdivided into a series of multiatomic (quasimolecular) pieces. The time-averaged Gaussian probability distribution of each piece projected onto the bilayer normal can be described by

$$n_i(z) = (N_i/A_i\sqrt{\pi})\exp[-(z - Z_i/A_i)^2], \quad (1)$$

where $n_i(z)$ is the fraction of the piece located at position Z_i with $1/e$ half-width A_i (Fig. 1). 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 Eq. 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 per 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 Eq. 1 by, respectively, the neutron scattering length b_{ni} or x-ray scattering length b_{xi} of piece i so that the scattering length per unit length is

$$\rho_{ji}^*(z) = b_{ji}n_i(z), \quad (2)$$

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

$$\rho_j^*(z) = \sum_{i=1}^p \rho_{ji}^*(z), \quad (3)$$

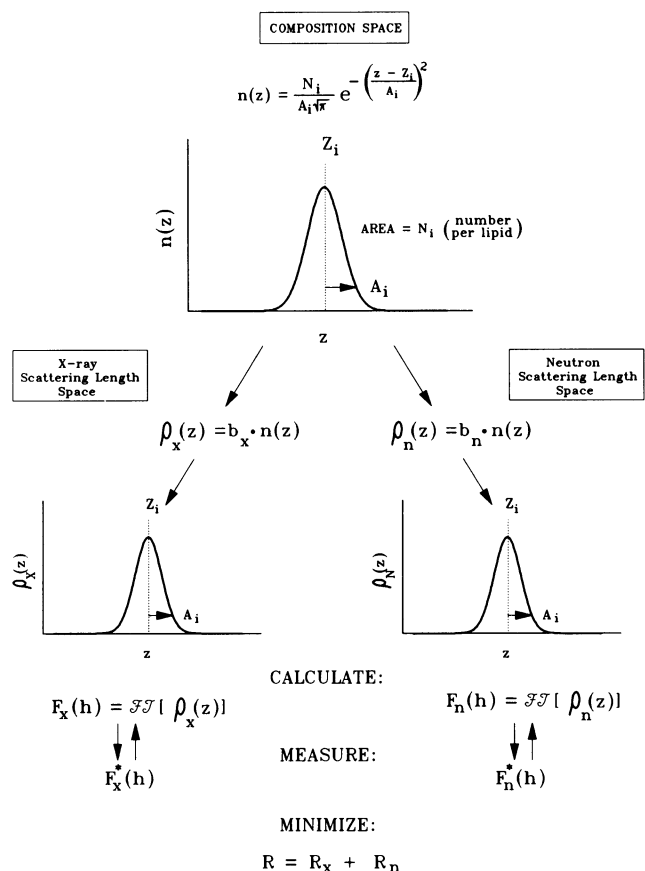


FIGURE 1 Summary of the composition-space refinement method. The basic strategy is to determine probability or occupancy functions $n_i(z)$ which describe the time-averaged transbilayer distribution of various parts of the hydrated lipid molecule. Scaling the functions by their neutron and x-ray scattering 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 quasimolecular models.

where p is the number of quasimolecular 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 composition space and scattering space representations (Eqs. 1–3) provide a fully resolved image of the average bilayer structure projected onto the bilayer

normal. This one-dimensional structure must be on a corresponding one-dimensional absolute scale to depict accurately the packing of the bilayer components along the bilayer normal. If the bilayer profile, given as occupancy or scattering length per unit length, is divided by the average area per lipid S , then the profile is converted to a “per volume” scale. Previously, White and Jacobs (1989) referred to “per unit length” profiles as being on the “relative absolute scale”; the profiles obtained by dividing by S yield profiles on “the absolute scale.” The structures obtained in the composition space refinements will generally use the relative absolute scale. The area S is not obtainable from lamellar diffraction and is most readily determined from absolute specific volume measurements (Nagle and Wiener, 1988, 1989).

An important aspect of the composition space representation of a bilayer is that any extensive property can be represented by multiplying Eq. 1 by the appropriate extensive variable for piece i . This is a manifestation of the property that an extensive variable of a system is obtainable from the superposition of the extensive variables of its constituent subsystems (Pippard, 1961). The mass is of particular importance. If μ_i is the mass of piece i , then the mass per unit length $m_i(z)$ is

$$m_i(z) = \mu_i n_i(z), \quad (4)$$

and the total mass per unit length at a point z in the bilayer is given by

$$M(z) = \sum_{i=1}^p m_i(z). \quad (5)$$

A slab of bilayer of thickness Δz and average molecular area S will have a mass density $D(z) = [M(z) \cdot \Delta z] / (\Delta z \cdot S)$ at z or

$$D(z) = M(z)/S. \quad (6)$$

The average mass density, which we shall henceforth simply refer to as “the density,” is given by

$$D_0 = (2/Sd) \int_0^{d/2} M(z) dz. \quad (7)$$

Therefore, if the density of the bilayer is determined independently, it is possible in principle to determine the molecular packing of the bilayer. If v_i is the molecular volume of piece i then at any point the mass density at z must be given by

$$D(z) = M(z) / \sum_{i=1}^p v_i n_i(z). \quad (8)$$

Because this equation requires estimates of all of the molecular volumes of the quasimolecular fragments at point z , it may be of limited applicability except at those

locations within the bilayer where one can be certain that there are only one or two pieces.

The main purpose of presenting Eqs. 7 and 8 is to emphasize that the scattering length functions of Eq. 3 are on an absolute scale (*vide ut infra*). The molecular packing is well defined at a given point in phase space (fixed temperature, pressure, composition) so that the composition space distributions $n_i(z)$ are well determined. The object of the diffraction experiment is to determine these distributions. This is done by determining the structure factors from the experimentally measured low-angle intensities and comparing them with the structure factors obtained by Fourier transformation of Eq. 3:

$$F_j(h) = 2 \sum_{i=1}^p b_{\mu} N_i \exp(-(\pi A_i h/d)^2) \cdot \cos(2\pi Z_i h/d). \quad (9)$$

If $F_j^*(h)$ are the experimental structure factors scaled to the relative absolute scale, then nonlinear minimization is used to determine the parameters A_i and Z_i of Eq. 1 which minimize the joint crystallographic R -factor defined here as

$$R = \sum_{j=n,x} R_j, \quad (10)$$

where

$$R_j = \sum_h ||F_j(h)| - |F_j^*(h)|| / \sum_h |F_j^*(h)|. \quad (11)$$

The experimental structure factors $f_j(h)$ actually measured are on an arbitrary scale but they can be converted to absolute structure factors by an appropriate instrumental scaling factor k_j :

$$f_j(h) = k_j F_j^*(h). \quad (12)$$

The determination of the scaling factors k_j permits one to determine (King et al., 1985; Jacobs and White, 1989) the scattering length density function $\rho_j^*(z)$ (Eq. 3) by means of

$$\rho_j^*(z) = (2/d) \sum_{i=1}^p b_{\mu} N_i + (2/dk_j) \sum_{j=1}^{h_{\max}} f_j(h) \cos(2\pi h z/d). \quad (13)$$

The first term of the equation is the average scattering length density per unit length of the bilayer and the second term describes the fluctuations in scattering length about this average across the bilayer. Omission of the first term and the scaling factor k_j in the second term yields the oft-cited ‘‘arbitrary scale’’ density profile. In principle, h_{\max} is a number without bound. In practice, however, as discussed extensively in the first paper of

this series, h_{\max} is usually no larger than ~ 25 . For fluid bilayers, h_{\max} is typically between 5 and 10. As discussed earlier, $\rho_j^*(z)$ is on the relative absolute (per unit length) scale. Using the earlier notation of Jacobs and White (1989),

$$\rho_j^*(z) = S \cdot \rho_j(z), \quad (14)$$

where $\rho_j(z)$ is the profile on the absolute (per unit volume) scale.

Two crucial questions remain unanswered. First, how many quasimolecular pieces are required and, second, what are the compositions of the pieces? These issues are discussed in the next two sections.

THE NUMBER OF QUASIMOLECULAR COMPONENTS

In the first paper of this series, model calculations demonstrated that d/h_{\max} is a reasonable estimate of the widths of the quasimolecular pieces. That is, a significant scattering region of the bilayer is expected to be representable by a quasimolecular component with a $1/e$ full-width $2A$ given by

$$2A \approx d/h_{\max}. \quad (15)$$

A simple calculation demonstrates that a complete and accurate model structure requires $\sim h_{\max}$ quasimolecular Gaussian components. In an experiment, each diffraction order represents information necessary for a description of the bilayer structure. As the complexity of the structure increases with an increase in the number p of significant scatterers, the greater complexity of the structure is manifested by a larger number h_{\max} of diffraction orders. We use a simple model to derive the relation between p and h_{\max} .

Consider a half-unit cell of width $d/2$ with a total scattering length B per lipid molecule (including water and any other molecules). Let this half-unit cell be represented by p Gaussians such that the scattering lengths in the lattice are uniformly distributed among them. The average scattering length of each Gaussian in the lattice will therefore be

$$b_i \equiv b = B/p. \quad (16)$$

For a uniform distribution of these p Gaussians within the half-unit cell, their positions are given by

$$Z_i = (i - 1)d/2p, \quad (17)$$

where $i = 1, \dots, p$. Let the widths of these uniform Gaussians be identical:

$$A_i \equiv A. \quad (18)$$

In this simple model, an arbitrary value of A will yield half-unit cells of total scattering length B so that, in principle, A can take on any positive value. Physically, however, one expects A to be restricted by bilayer packing constraints and to be related to the total number p of Gaussians. A simple and reasonable supposition is that the width $2A$ of a Gaussian is proportional to the region of width $d/2p$. We therefore write

$$2A = Kd/2p, \quad (19)$$

where K is a constant of proportionality. Substituting d/h_{\max} for $2A$ (from Eq. 15) leads to a simple relation between p and h_{\max} ,

$$p = K'h_{\max}. \quad (20)$$

The constant of proportionality K' has absorbed the factor of 2 from Eq. 19 as well as the approximation of Eq. 15. A value of K' cannot be easily developed from first principles; we therefore estimate it from germane structural results in the literature. Hitchcock et al. (1974) solved the structure of dilauroylphosphatidylethanolamine cocrystallized with acetic acid. There are $p = 43$ atoms in the half-unit cell and $h_{\max} = 44$ reflections (0, 0, l) were observed, so $K' \approx 1.0$. The crystal structure of n -octadecane including hydrogens was refined by Nyberg and Lüth (1972) from the data of Hayashida (1962). There are $p = 28$ atoms in the half-unit cell and $h_{\max} = 29$ reflections (0, 0, l) were observed which again gives $K' \approx 1.0$. In a study of liquid-crystalline bilayers formed from lipids extracted from human erythrocytes, Rand and Luzzati (1968) fitted a single $p = 2$ Gaussian model to a series of dispersions at different hydrations where $h_{\max} = 4-9$ orders were observed for the various samples. The result is a range of $0.2 \leq K' \leq 0.5$. At the other extreme of possible K' values, Sakurai et al. (1977) obtained $h_{\max} = 25$ orders from oriented egg phosphatidylcholine. They used these data to fit an atomic model consisting of $p = 52$ atoms, yielding $K' \approx 2.1$.

These estimates indicate that a range of scale factors K' can be obtained depending upon the specific membrane system under consideration as well as the completeness of the structural analysis. However, based upon our experiences (Wiener, M., and S. White, manuscript in preparation) with liquid-crystalline dioleoylphosphatidylcholine (DOPC), we state the following "rule-of-thumb" for the number of quasimolecular components: the number of Gaussians p is approximately equal to the number h_{\max} of observed lamellar diffraction orders. The two crystallographic examples above support the rule-of-thumb but we stress that it is an approximate relation that is likely, in specific cases, to vary between the upper and lower bounds calculated above. Whereas $p \approx h_{\max}$ 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.

COMPOSITION OF THE QUASIMOLECULAR COMPONENTS: PARSING STRATEGIES

There are many ways to divide a lipid molecule into p fragments but two important guidelines simplify the process. The first guideline, discussed in the previous section, is to parse the hydrated molecule into $p \approx h_{\max}$ pieces that have widths $2A_i \approx d/h_{\max}$. The second guideline is inherent in the composition space refinement method. Namely, the positions Z_{ji} 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 (Eq. 2) 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 as described in detail in the first paper (Wiener and White, 1990). The parsing must be done largely by trial and error in specific cases as we will describe in detail later for DOPC multilayers (Wiener, M., and S. White, manuscript in preparation). However, the general principles can be revealed by considering the following hypothetical quasimolecular models derived from phospholipid crystal structures.

In previous applications of atomic-level crystal structures or models, the atomic positions and Debye-Waller factors were adjusted to obtain the best fit to experimental data for crystalline (Hitchcock et al., 1975; Sakurai et al., 1977; Dorset, 1987) or liquid-crystalline (Franks, 1976) bilayers. We use phospholipid crystal data differently in this calculation. The central tenet of the composition space quasimolecular refinement is that fragments of approximate width d/h_{\max} are the best representation of liquid-crystalline bilayers. We use the crystallographic data for testing the acceptability of various groupings of atoms in composition space by means of simple center-of-scattering (center-of-mass) calculations. Each atom in the crystal structure has a position z_q and scattering length b_{jq} . For quasimolecular component i containing n_i atoms, the center-of-scattering Z_{ji} in neutron or x-ray scattering length space is given by the expression

$$Z_{ji} = \sum_{q=1}^{n_i} b_{jq} z_q / \sum_{q=1}^{n_i} b_{jq}. \quad (21)$$

If the neutron and x-ray centers-of-scattering of a quasimolecular piece differ for a particular parsing, then the composition space profile may not be a satisfactory representation of the piece, ignoring thermal disorder.

As a specific example, we consider two parsings based upon the crystal structure of DMPC (Pearson and Pascher, 1979; coordinates in Brookhaven Protein Data Base). The atomic coordinates are projected onto the bilayer normal to arrive at values of z_q . Two molecular conformers (labeled here I and II) exist in the crystal and we treat them as separate cases. Hydrogens are placed at their corresponding carbon positions in the structure. For individual atoms, the neutron scattering lengths are from Sears (1986) and the x-ray scattering lengths for low-angle lamellar diffraction are given by the atomic number multiplied by e^2/mc^2 (Warren, 1969). We restrict our attention solely to a comparison of the centers-of-scattering Z_{ji} of the quasimolecular fragments as seen by neutron and x-ray diffraction because the calculation of the widths of the quasimolecular regions requires additional assumptions about atomic diameters and their ranges of motion. Information from these

more complicated and speculative assumptions adds little to the present discussion.

As frequently stated, liquid-crystalline bilayers at partial hydration yield 5–10 lamellar diffraction orders; we observe eight orders from DOPC. As an initial model, consider a quasimolecular structure with $p = (h_{\max}/2) = 4$ components which is in the lower range of the number of Gaussians derived in the previous section. The parsing chosen for the model is shown as Model A in Fig. 2. The CH_2 region is represented as a single piece because the region is quite homogeneous and the discrimination of individual methylenes in chain-melted liquid-crystalline phases is physically unlikely. The terminal methyl groups are considered as a separate and distinct piece because of the characteristic low-density “methyl trough” seen even in highly fluid liquid-crystalline bilayers. Moving toward the headgroup, the third piece (symbolized $\text{C} = \text{O} + \text{GLYC}$) consists of the carbonyl groups and the glycerol backbone which can be considered as the transition region between the alkyl chains and the zwitterionic headgroup. The fourth piece ($\text{PO}_4 + \text{CHOL} + \text{W}$) encompasses the phosphate,

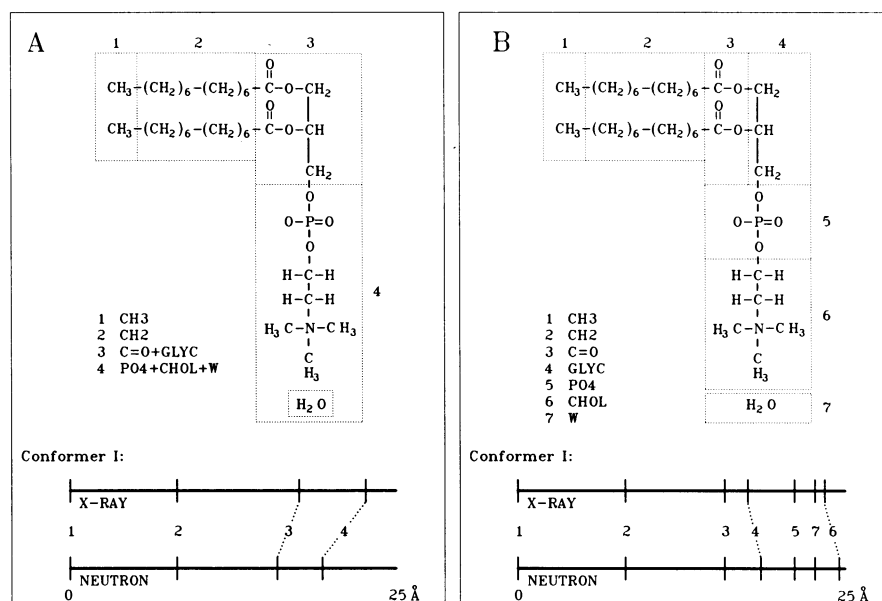


FIGURE 2 Two hypothetical models for the parsing of the atoms of dimyristoylphosphatidylcholine (DMPC) into quasimolecular pieces based upon crystallographic coordinates (Pearson and Pascher, 1979) and physicochemical considerations. Neither model takes into account the thermal disorder expected of liquid-crystalline DMPC. There are two conformers of DMPC in the crystal lattice. The relative positions indicated at the bottoms of figures are for conformer I (Table 1). (Model A.) In this model the atoms of the half-unit cell are parsed into four pieces designated as CH_3 , CH_2 , $\text{C} = \text{O} + \text{GLYC}$ (carbonyls and glycerol backbone) and $\text{PO}_4 + \text{CHOL} + \text{W}$ (phosphocholine headgroup and associated waters of hydration). A simple center-of-scattering calculation (Eq. 21 and Table 1) yields the apparent x-ray and neutron diffraction positions of the pieces shown schematically at the bottom of the figure. (Model B.) This model has seven pieces and is derived from Model A by the further parsing of $\text{C} = \text{O} + \text{GLYC}$ into separate $\text{C} = \text{O}$ and GLYC pieces and the $\text{PO}_4 + \text{CHOL} + \text{W}$ into PO_4 , CHOL , and W pieces. The relative positions of the x-ray and neutron centers-of-scattering are in much better agreement as shown at the bottom of the figure (see Table 1).

choline, and associated waters of hydration. This parsing is influenced by a melange of physicochemical information including the schematic formula of the molecule, CPK models, existing membrane diffraction data, and common sense.

The neutron and x-ray centers-of-scattering of the four quasimolecular components of Model A are listed in Table 1 and shown schematically in Fig. 2 for conformer I. The CH₃ and CH₂ regions, being quite homogeneous, have identical centers in x-ray and neutron space whereas the C = O + GLYC and PO₄ + CHOL + W regions do not. The differences of 0.5 Å for C = O + GLYC and 3.3 Å for PO₄ + CHOL + W are very significant based upon our study of resolution in the first paper (Wiener and White, 1990). These differences arise from differences in the relative sizes of the scattering lengths of the atoms (particularly hydrogens) in each scattering space. We note that these positions can be viewed as those that might be obtained from quasimolecular models independently fit to each data set.

Now consider the more finely parsed Model B of Fig. 2 in which C = O + GLYC is divided into separate C = O and GLYC moieties and PO₄ + CHOL + W into PO₄, CHOL, and W regions. A comparison of the neutron and x-ray centers-of-scattering in this case (Table 1 and Fig. 2 [Model B]) reveals much closer agreement between the positions of the C = O, PO₄,

and W regions. The positions of the GLYC and CHOL pieces, however, remain significantly different because of the relative difference in neutron and x-ray scattering lengths of the hydrogens (hydrogen has a *negative* neutron scattering length). Two considerations may ameliorate the differences. First, the hydrogens were placed at the same positions as their corresponding carbons in the crystal structure projections. Shifting the centers-of-scattering of some of the hydrogens in GLYC and CHOL several tenths of an angstrom can wipe out these differences in position. Second, whereas the crystal structure is useful as a starting point for fluid bilayer structure, there is no a priori justification for assuming that the specific conformers in a crystal structure are equivalent to the average structures in liquid-crystalline bilayers (Strenk et al., 1985). The actual average structure of a thermally disordered liquid-crystalline bilayer may not have the differences in centers-of-scattering seen in the crystal structure-based calculation. This model has $p = 7$ pieces consistent with the rule-of-thumb that $p \approx h_{\max}$.

These model calculations emphasize the necessity of understanding the thermal disorder which is an essential and important feature of fluid bilayers. While we cannot know a priori the details of the thermal disorder of a fluid DMPC bilayer, it is useful to extend Model B to include thermal disorder. For this purpose, assume the C = O and GLYC pieces of conformer I have the mean positions given in Table 1 and treat them as having Gaussian distributions with 1/e half-widths of 3 Å. The scattering length distributions in x-ray and neutron space are shown in Fig. 3, A and B, respectively. The relative scattering lengths of the two pieces in the two scattering length spaces are striking; the relative contribution of GLYC for neutrons is trivial. This means that a neutron diffraction experiment can locate accurately the position of the C = O but not the GLYC. In an x-ray experiment, on the other hand, the GLYC makes a significant contribution. The two Gaussians for the two pieces in each scattering length space have been summed together in Fig. 3 C. The positions of the summed Gaussians are, as expected, precisely the same as the positions calculated for the C = O + GLYC of conformer I in Model A (data not shown). These results demonstrate a major strength of the composition space joint refinement method: neutrons reveal the position and width of the C = O group which can be used to isolate the GLYC position and width from the x-ray experiment. Fig. 3 C illustrates the problem of converting x-ray data to neutron data and vice versa. If one were to assume, for example, that the neutron result could be transformed to x-ray space by using the neutron Gaussian's position and width in Eq. 2, a serious error would result. Even though the positions of the summed Gaus-

TABLE 1 Positions of quasimolecular pieces of dimyristoylphosphatidylcholine (DMPC) for two models calculated on a center-of-scattering basis from the crystalline coordinates of Pearson and Pascher (1979)

		Conformer I		Conformer II	
		Z _{ni}	Z _{xi}	Z _{ni}	Z _{xi}
		Å	Å	Å	Å
MODEL A*					
i = 1	CH ₃	-1.17	-1.17	-2.76	-2.76
i = 2	CH ₂	7.00	7.00	5.28	5.28
i = 3	C = O + GLYC	14.76	15.28	13.41	13.87
i = 4	PO ₄ + CHOL + W	18.00	21.27	17.33	18.56
MODEL B* (Hydrocarbon region same as for Model A)					
i = 3	C = O	14.67	14.67	13.32	13.35
i = 4	GLYC	17.41	16.42	15.96	14.86
i = 5	PO ₄	20.00	19.96	17.90	17.87
i = 6	CHOL	23.37	22.27	20.00	19.21
i = 7	W	21.55	21.55	—	—

The models are described in greater detail in Fig. 2. Two conformers are observed in the crystal lattice and are designated here I and II. The positions of the pieces are calculated by means of Eq. 21 and are given in angstroms (1 Å = 0.1 nm). The subscripts n and x refer to neutron and x-ray respectively. The positions are determined by the relative scattering lengths of the constituent atoms in the pieces.

*Abbreviations: CH₃: 2 methyl groups; CH₂: 24 methylene groups (12/chain); C = O: 2 carbonyl groups; CHOL: 1 choline group; GLYC: 1 glycerol backbone; PO₄: 1 phosphate group; W: water.

sians differ by only 0.5 Å, this error is easily detected (Wiener and White, 1990). Further, the 1/e half-width of the summed x-ray Gaussian is ~3.4 Å whereas the neutron Gaussian is little different from the C = O Gaussian; this would create a significant error in the apparent thermal motion of the C = O + GLYC piece.

These results, based upon crystal structures, illustrate some of the ideas that motivate the parsing of a membrane into a composition space model. In the actual development of composition space representations for a particular membrane system, crystal structures are not essential and the presence of atomic level information

may even obfuscate the issue. In our investigation of DOPC (Wiener, M., G. King, and S. White; Wiener, M., and S. White [both manuscripts in preparation]), physicochemical information rather than crystallographic data provides the main source of information for the refinement. Molecular models can be of use in model development but the Gaussian positions Z_i and widths A_i of a specific model must be ultimately determined from fitting the model's structure factors (Eq. 9) to experimental data by means of Eq. 11.

DEGREES OF FREEDOM IN COMPOSITION SPACE REFINEMENT

A crucial aspect of any modeling procedure is that the models cannot be underdetermined with respect to the experimental data. It is thus important to consider the number of degrees of freedom required of a composition space model and to compare this number, n_f , with the amount of information obtainable in typical experiments. Each Gaussian requires three parameters: position Z_i , 1/e half-width A_i , and composition N_i . For a structure consisting of p Gaussians, there are therefore at least $n_f = 3p$ degrees of freedom. Because the joint refinement procedure requires that the neutron and x-ray diffraction data be on absolute scales, the instrumental scaling factors k_n and k_x (cf. Eq. 12) increase n_f to $3p + 2$. Use of the approximate relation $p \approx h_{\max}$ yields $n_f \approx 3h_{\max} + 2$. This result means that a complete and accurate structure *cannot* be obtained entirely on the basis of the experimentally determined neutron and x-ray structure factors ($2h_{\max}$ data points) without additional information. Our approach to the problem of obtaining the additional information necessary for the

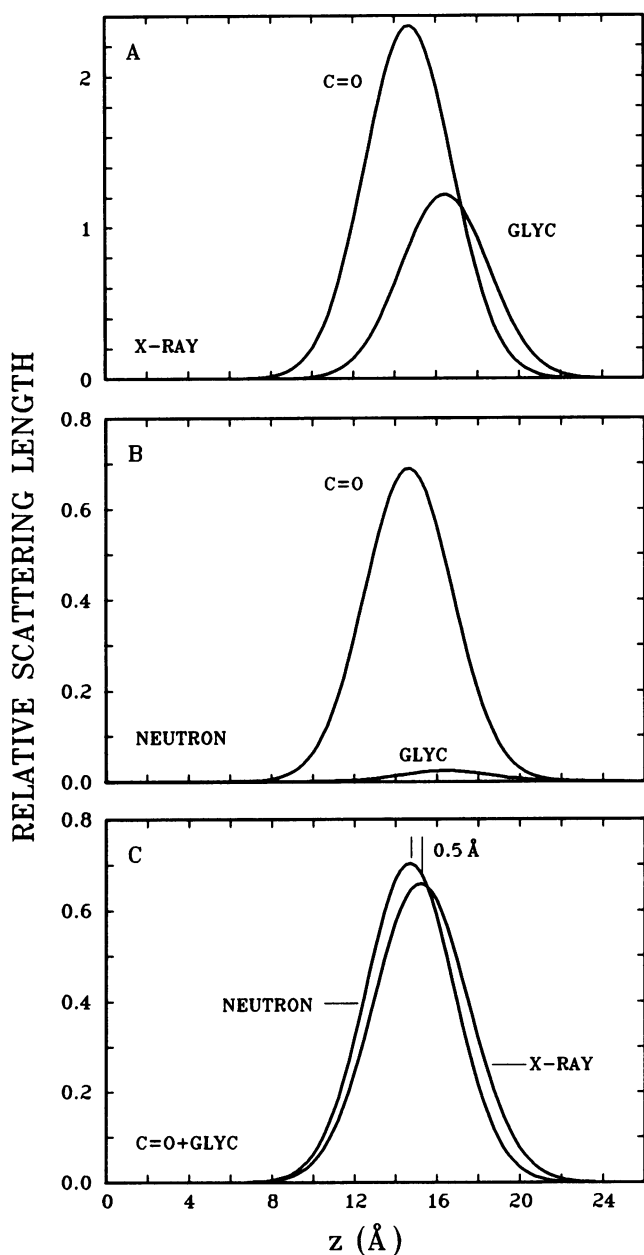


FIGURE 3 Representations of the carbonyl and glycerol pieces of DMPC as thermally disordered Gaussian distributions. The positions of the carbonyls (symbolized C = O) and glycerol (symbolized GLYC) are those of conformer I in *Model B* (Fig. 2 and Table 1). The 1/e half-widths of the distributions have been set arbitrarily to 3 Å because this width is expected for fluid bilayers producing seven or eight orders of diffraction. The areas of the Gaussians represent the total relative x-ray or neutron scattering lengths of the pieces. (A) Scattering length distributions for x-rays. Note that both the C = O and GLYC moieties are significant scatterers. (B) Scattering length distributions for neutrons. Here the scattering by the GLYC is trivial compared to that of the C = O so that the latter dominates the scattering. (C) The two Gaussians in A and B have been summed together. The summed curves themselves are very close to Gaussian. The positions of the summed curves are those expected for model A (Fig. 2). This figure shows the problem of inappropriately converting between x-ray and neutron "space." If one were to simply rescale the x-ray produced Gaussian, for example, to produce one for neutron diffraction, both the positions and widths would be inconsistent with neutron observations. See text.

joint refinement of the structure of DOPC bilayers will be described in detail in a later publication (Wiener, M., and S. White, manuscript in preparation). However, because of the importance of properly specifying models, we present here a brief summary of our approach. The obvious goal is to reduce the degrees of freedom of the model and/or increase the number of experimentally determined quantities. The strategies for accomplishing this goal fall into three broad categories: parsing, physicochemical, and specific labeling.

Parsing

We showed above and in the first paper (Wiener and White, 1990) that certain regions of the bilayer will be more determinable than others as a consequence of their relative contributions to the total scattering and amounts of thermal motion. For instance, the carbonyl groups are the major contributors to neutron scattering and the phosphate group the major contributor to x-ray scattering. It is reasonable to account for this fact in the parsing so that the combined diffraction data can be used to locate these features accurately and uniquely in the joint refinement. At the opposite extreme, the regions of the bilayer which scatter less distinctly because of thermal disorder and/or relative scattering length, specifically the methylene region, require a different approach. A specific CH_2 group cannot be uniquely ascribed to a particular position or even region of the bilayer. In our earlier discussion of parsing strategies we represented this region as a single piece. However, the distribution of methylenes in a fluid bilayer is more complex than a single Gaussian function (Gruen, 1980; Cantor and Dill, 1984) and in our analysis of DOPC bilayers three Gaussians are necessary to describe the shape of the distribution. Because of the inherent disorder, one can relax the requirements for a unique or optimal solution for each Gaussian. It turns out that the sum of these three Gaussians yield a unique total methylene profile even though there are many three-Gaussian combinations that yield the same total methylene envelope. That is, the unique and physically meaningful CH_2 distribution is degenerate in the Gaussian basis set of quasimolecular models. Whereas this degeneracy affects the uniqueness of CH_2 parsing, satisfactory terminal methyl and headgroup models can be determined because of their particular scattering characteristics.

Physicochemical

The primary use of this approach has been limited to the determination of unit-cell composition and bilayer mass density although other uses can be envisaged. In particu-

lar, we determined the number of waters hydrating the headgroup (White et al., 1987; Jacobs and White, 1989). This provides N_i in Eq. 1 for the water quasimolecular piece and aids in the determination of the water distribution and the neutron instrumental constant.

Specific labeling

Neutron diffraction measurements of samples hydrated with different proportions of D_2O and H_2O provide the phases of the neutron structure factors (Blasie et al., 1975; Worcester and Franks, 1976; Jacobs and White, 1989) and the neutron scale factor k_n . In addition, the difference structure factors obtained from various $\text{D}_2\text{O}/\text{H}_2\text{O}$ compositions yield the water distribution which is thus determined independently of the joint refinement. Neutron diffraction of DOPC specifically deuterated at the double-bond position allowed determination of the C = C distribution within the bilayer and provided a check on k_n as well. We have determined the x-ray scale factor k_x from diffraction of mixtures of DOPC with a specifically brominated phosphatidylcholine. The use of halogenated molecules for the absolute scaling of membrane diffraction data, first utilized by Franks et al. (1978), will be described in our next paper of this series (Wiener, M., G. I. King, and S. White; Wiener, M. and S. White, manuscripts in preparation).

The requirement that a single structure fit neutron and x-ray data placed severe constraints on parsing, with one successful model among 30 or more posited structures. We note that if the water and double-bond distributions had not been independently determined, they would have been combined in some way with the h_{max} pieces of the joint refinement. Because the methylenes were modeled as three Gaussians with a fixed total area, the parsing reduced n_f by $h_{\text{max}} - 2$ (instead of by h_{max}) to $2h_{\text{max}} + 4$. The independent determination of the two scale factors reduced n_f to $2h_{\text{max}} + 2$. The terminal methyl must be located at $Z = 0$ and we have devised a method for the determination of its width from a combination of the neutron and x-ray structure factors prior to the full joint refinement (Wiener, M., and S. White, manuscript in preparation). The net result is that there are $2h_{\text{max}}$ degrees of freedom and $2h_{\text{max}}$ structure factors. However, because the envelope of the sum of the methylene Gaussians is preserved and because this envelope can be described by many different three-Gaussian combinations, it is feasible to fix the number of methylenes in each of the three Gaussians; n_f is then reduced by 2 to $2h_{\text{max}} - 2$.

In the determination of other bilayer structures, different combinations of known and unknown information can occur and we briefly discuss some of the more likely possibilities. If the hydration number is not known,

$n_f = 2h_{\max} - 1$ and the problem is still completely determinable from the available data. Any additional components of the unit cell that are not determined before the structural determination will each add a degree of freedom. For instance, if a peptide-lipid or binary-lipid mixture of unknown stoichiometry were investigated, the degrees of freedom increase. Another likely possibility is that one or both of the instrumental constants k_n and k_x are not determined. Ignorance of one scaling factor only adds another degree of freedom ($n_f = 2h_{\max}$) so the problem is, in principle, determinable from joint refinement. If both are unknown, the structure is under-determined from the available diffraction data. If either scale factor is unknown, the terminal methyl distribution cannot be obtained from the neutron and x-ray diffraction data sets, so another degree of freedom is added.

ERROR ANALYSIS

We have demonstrated that the number of parameters of a quasimolecular structure is barely less than or equal to the number of data points. This places unique demands on the error analysis methods that are used to determine the best structure as well as the uncertainties of individual structural parameters. Conventionally, a χ^2 merit function is minimized. χ^2 in standard least squares minimization is the sum of the squares of the differences of model and data, divided by the number of degrees of freedom in the problem. The degrees of freedom, used to distinguish the best of a group of models with differing numbers of parameters, is the difference between the number of data points and parameters. However, this can be zero in the quasi-molecular refinement. We avoid the possible singularity by minimizing instead the joint crystallographic R-factor (Eq. 11). As discussed in the first paper (Wiener and White, 1990), a set of measured structure factors $F^*(h)$ has an overall uncertainty or "self" R ", R_s . A composition space structure that yields neutron and x-ray R-factors less than or equal to the respective R_s values is a satisfactory jointly refined structure. In theory, several models with differing numbers of parameters could all provide satisfactory fits and there is no justification (other than Occam's razor) for choosing one model over another. However, as noted earlier, only one composition space structure among more than thirty tested satisfied both neutron and x-ray data sets. Most unsuccessful models fitted only one data set adequately, some fit neither data set well, and a few did not converge at all. Our experience to date indicates that locating many satisfactory structures with a wide range in numbers of parameters is not likely.

The robustness of a successful model and the uncer-

tainties of its parameters can be determined in several ways. One way to test robustness is to determine if there is any sensitivity of the final model parameters to the choice of initial parameters in the nonlinear minimization. We find that the minimization converges to the same solution over a range of starting parameters and diverges outside of this range, a common feature of any minimization routine. The uncertainties of parameters obtained in nonlinear minimization are often obtained from the elements of the covariance matrix (Press et al., 1986). However, this approach can underestimate the confidence intervals (Johnson, 1983). We prefer to estimate parameter uncertainties in two ways. In the first, each parameter in a solution is systematically varied from its best value and the joint crystallographic R-factor is calculated at each new value. A plot of R-factor versus parameter value traces out a "basin" with its minimum at the best value of that parameter; the range of parameter values with $R < R_s$ serves to define the confidence interval of the parameter. The other approach derives from the fact that each experimental structure factor $F_j^*(h)$ has an associated experimental uncertainty that can be used to define a normal distribution of structure factors. A statistical Monte Carlo procedure can be used to generate mock data sets from these distributions (Press et al., 1986) and the composition space model fit to each of these "data" sets. The distributions of parameter values obtained from fitting many sets of "noisy" mock data provides the average values and variances of structural parameters. We will describe in detail the determination of these uncertainties in a later paper (Wiener, M., and S. White, manuscript in preparation).

DISCUSSION

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 (Franks and Lieb, 1981). At low scattering angles, x-ray scattering length is proportional to atomic number. Neutron scattering length, on the other hand, depends upon nuclear interactions which are not related to atomic number in a simple way. Generally, the neutron scattering lengths of most atoms differ by no more than about a factor of two. The most important exception is hydrogen which has a *negative* scattering length. The utility of combining neutron and x-ray diffraction data in the joint refinement procedure arises from the nontrivial differences between their respective scattering lengths. 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.

The neutron and x-ray centers-of-scattering in small crystals can differ at individual atoms because the center of electron density need not coincide with the atomic nucleus (Coppens, 1967, 1974). In these instances, different atomic-resolution structures are obtained from the two diffraction experiments which yield important information on the displacements of the electron clouds relative to the nuclei. In contrast, the liquid-crystalline bilayer has a highly thermally disordered unit cell. On the time-scale of a diffraction experiment, the neutron and x-ray centers-of-scattering of individual atoms in a fluid bilayer cannot be distinguished. We demonstrated in the first paper that the typical width of significant scattering regions of the bilayer, given approximately by d/h_{\max} , is much greater than that of single atoms. This leads to the basic principle of the composition refinement method: a single real-space distribution of matter, the composition space profile, must exist which gives rise to both sets of diffraction data and other extensive properties of the bilayer as well (Fig. 1). The composition space profile is a quasimolecular model with each Gaussian distribution representing a thermally disordered fragment of the molecule. Instead of neutron or x-ray scattering density, the composition space profile is in the more fundamental units of number-density or occupancy-per-unit length.

Two methods can now be used to obtain the detailed composition space structure of a liquid-crystalline bilayer. The traditional method is to prepare many lipid isomorphs, each specifically labeled, and perform diffraction upon each one of them to locate the labels and therefore the quasimolecular pieces. But this is an arduous task accomplished only by Büldt and co-workers (Büldt et al., 1978; Büldt and Seelig, 1980; Mischel et al., 1987) under restricted conditions. We have presented here a more feasible alternative which we hope will lead to a better understanding of a broader range of fluid bilayer systems.

SUMMARY

Multilamellar arrays of liquid-crystalline bilayers can form nearly perfect one-dimensional lattices of thermally disordered unit cells. The number of observed diffraction orders is a direct indication of the number and the widths of the Gaussian quasimolecular regions comprising the bilayer. The joint refinement technique

provides a fully resolved image of the low-resolution thermally disordered bilayer. However, the combination of the thermal disorder and the one-dimensional structure fundamentally makes the structure barely determinable from the observed data. The one-dimensional lattice limits additional information normally obtained from the remaining two dimensions in three-dimensional crystals. In macromolecular crystallography the structure is, in principle, overdetermined from diffraction data (Hauptmann, 1986). Additional information from physicochemical measurements, parsing strategies, and limited specific labeling will always be necessary to determine fully resolved images of liquid-crystalline membranes. Real-space physicochemical refinement ("outside information") is also used for macromolecular crystal-structure analysis (Hendrickson, 1985); very few protein structures are solved solely from diffraction data. The joint refinement technique for bilayers and the use of quasimolecular structural models can be viewed as an effort to develop and further the theory and practice of *liquid-crystallography*.

We thank Dr. Steven L. Blechner for providing the crystallographic coordinates of DMPC.

This work was supported by grants from the National Science Foundation (DMB-880743) and the National Institute of General Medical Sciences (GM-37291).

Received for publication 27 July 1990 and in final form 24 September 1990.

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