Structure of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of x-ray and neutron diffraction data

II. Distribution and packing of terminal methyl groups

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ABSTRACT We continue in this paper the presentation of theoretical and experimental methods for the joint refinement of neutron and x-ray lamellar diffraction data for the analysis of fluid (L_α phase) bilayer structure (Wiener, M. C., and S. H. White. 1991 *a*, *b*, *c*. *Biophys. J.* 59:162–173 and 174–185; *Biochemistry.* 30:6997–7008; Wiener, M. C., G. I. King, and S. H. White. *Biophys. J.* 60: 568–576). We show how to obtain the distribution and packing of the terminal methyls in the interior of a fluid dioleoylphosphatidylcholine bilayer (66% RH) by combining x-ray and neutron scattering-length transbilayer profiles with no a priori assumptions about the functional form of the distribution. We find that the methyls can be represented by a Gaussian function with 1/e-halfwidth of 2.95 ± 0.28 Å situated at the bilayer center. There is substantial mixing of the methyls and methylenes in the bilayer center. The Gaussian representation of the methyl distribution is narrower and has a different shape than predicted by several simulations of fluid bilayers (Gruen, D. W. R., and E. H. B. de Lacey. 1984. Surfactants in Solution, Vol. 1. Plenum Publishing Corp., New York. 279–306; de Loof, H., et al. 1991. *Biochemistry.* 30:2099–2133) but this may be due to the smaller area/lipid of our experiments and the presence of the double-bonds. Determination of the absolute specific volume of DOPC and an analysis of bulk alkane volumetric data over a range of hydrostatic pressures lead to estimates of methylene and methyl volumes at the bilayer center of 27 ± 1 $Å^3$ and 57.2 ± 3.6 $Å^3$, respectively. This result provides direct confirmation of the common assumption that the molecular packing of methyl and methylene groups in bilayers is the same as in bulk liquid alkanes.

INTRODUCTION

Structural information about the disposition of the hydrocarbon interior of fluid bilayers is important for understanding lipid-protein interactions (Chapman, 1968, 1983; Jacobs and White, 1989), protein–protein interactions in biomembranes (Popot and Engelman, 1990), and the microscopic properties of solutions (White et al., 1981; Dill et al., 1988; De Young and Dill, 1990). Even though we now have an extensive understanding of many aspects of fluid bilayers (Small, 1986), clear and detailed structural images have in the past been difficult to obtain except by laborious neutron diffraction studies (Büdt et al., 1979; Zaccai et al., 1979). Such images are, however, crucial for testing theoretical approaches to the determination of membrane structure and properties. The structures of micelles, monolayers, and bilayers have been investigated with a range of theoretical methods including mean-field (Schindler and Seelig, 1975; Gruen, 1980, 1985*a*, *b*; Gruen and de Lacey, 1984), lattice (Cantor and Dill, 1984*a*, *b*), generalized van der Waals (Meraldi and Schlitter, 1981*a*, *b*), Monte Carlo (Scott, 1977), and molecular dynamics calculations (van der Ploeg and Berendsen, 1982; Egberts and Berendsen, 1988; Pastor et al., 1991; de Loof et al., 1991). Much of this work has been concerned with the conformation of the phospholipid acyl chains so that direct experimental determination of acyl chain organization is of particular interest.

We consider in this paper the distribution and packing of the terminal methyls in the interior of a fluid bilayer. We show how a simple real-space combination of the absolute x-ray and neutron scattering density profiles yields the distributions of methyls and methylenes in the bilayer center. Although there is no a priori assumption about the shape of the distributions obtained by this direct real-space combination method, the transbilayer methyl distribution can be represented adequately by a Gaussian function centered in the middle of the bilayer with a 1/e-halfwidth of 2.95 ± 0.28 Å. We then compare the distribution with two published methyl distributions (Gruen and de Lacey, 1984; de Loof et al., 1991). The composition-space (Wiener and White, 1991*b*) profiles of the methyls and methylenes are then utilized to determine the volume of a terminal methyl in the bilayer interior. The analysis of alkane volumetric data (Dymond et al., 1979; Dymond et al., 1982) over a range of applied hydrostatic pressures is used to place bounds on

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the methylene volume of 27 ± 1 Å^3. Measurements of the absolute specific volume of DOPC, and the number of waters of hydration of DOPC at 66% RH (White et al., 1987) yield the average lipid cross-sectional area S = 59.3 ± 0.7 Å^2. Combining the methyl and methylene profiles with the methylene volume and lipid area enables the direct determination of a methyl volume of 57.2 ± 3.6 Å^3. This result provides direct confirmation of the common assumption that the molecular packing of methyl and methylene groups in bilayers is the same as in bulk liquid alkanes (Tardieu et al., 1973; Nagle and Wiener, 1988).

This paper is an outgrowth of our detailed investigation of methods for maximizing the structural information obtainable from lamellar diffraction of lipid bilayers. Two earlier papers established the theoretical foundations for the joint refinement of x-ray and neutron diffraction data in "composition space" to obtain fully-resolved images of fluid bilayer structure (Wiener and White, 1991*a*, *b*). Specific deuteration and specific halogenation were subsequently utilized to determine absolute neutron and x-ray structure factors of DOPC and the transbilayer distribution of the double-bonds (Wiener et al., 1991; Wiener and White, 1991*c*). These data and the terminal methyl distribution determined in the present paper will be used for the determination of the complete structure of DOPC (66% RH) that will be presented in the final paper of this series (Wiener and White, 1992).

METHODS

Experimental methods

The neutron and x-ray diffraction experiments have been described in detail (Jacobs and White, 1989; Wiener et al., 1991; Wiener and White, 1991*c*). The number of waters of hydration of DOPC at 66% RH was previously determined by White et al. (1987). These investigators also showed that lecithin and water mix ideally on a volumetric basis so that absolute specific volumes of lecithin in vesicles in excess water determined by neutral buoyancy centrifugation are equal to those determined at low hydration by direct volumetric and gravimetric measurements. In the present study, the absolute specific volume of DOPC was determined by H_2O/D_2O neutral buoyancy centrifugation of multilamellar vesicles using the method of Wiener et al. (1988).

Determination of CH_3 and CH_2 profiles

In the center of DOPC bilayers (-10 Å $\leq z \leq$ 10 Å) there are only three quasimolecular species: terminal methyls, methylenes, and double-bonds. However, the double-bond distribution is known from neutron diffraction of specifically deuterated DOPC ($-CD=CD-$, both chains) (Wiener et al., 1991). The double-bond is represented in "composition-space" by the equation $n(z) = (N/A\sqrt{\pi}) \exp[-[(z \pm Z)/A]^2]$ where Z is the position of the Gaussian, A the 1/e-halfwidth, and N the number of double bonds per lipid molecule (Wiener and White, 1991*b*). Composition-space distributions, which provide a direct image of the number per unit length or probability density of quasimolecular fragments, can be mapped to neutron or x-ray scattering-length space by scaling $n(z)$ by the appropriate neutron or x-ray scattering length (Wiener and White, 1991*b*). The scaled double-bond distributions can then be subtracted from x-ray and neutron scattering-length bilayer profiles to obtain double-bond-subtracted profiles, $\rho_X^*(z)$ and $\rho_N^*(z)$. The centers of the modified profiles contain only methyls and methylenes so that their distributions, $n_{CH3}(z)$ and $n_{CH2}(z)$ in units (Wiener and White, 1991*b*) of number per unit length, can be obtained from

$$\rho_N^*(z) = {}^N b_{CH3} \cdot n_{CH3}(z) + {}^N b_{CH2} \cdot n_{CH2}(z)$$
$$\rho_X^*(z) = {}^X b_{CH3} \cdot n_{CH3}(z) + {}^X b_{CH2} \cdot n_{CH2}(z), \quad (1)$$

where ${}^N b$ and ${}^X b$ denote the neutron and x-ray scattering lengths of methyls and methylenes.

RESULTS AND DISCUSSION

Fig. 1 depicts the absolute x-ray and neutron scattering density profiles of DOPC at 66% RH (Wiener et al.,

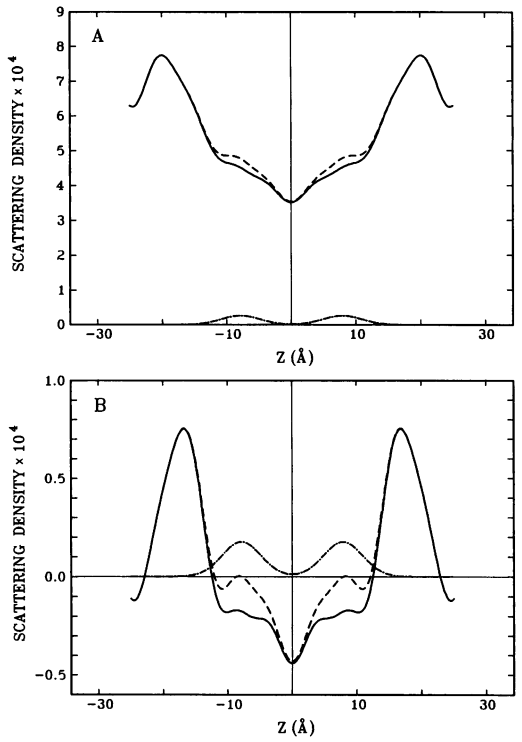

FIGURE 1 X-ray (*A*) and neutron (*B*) scattering-length density profiles used for the determination of the terminal methyl distribution of liquid crystalline (L_α phase) DOPC at 66% RH and 23°C. In *A* and *B*, the full profiles (eight diffraction orders) are represented by dashed curves (---), the double-bond profiles by broken curves (-----), and the double-bond-deleted profiles (i.e., full profile minus double-bond profile) by a solid curve (——). The central regions (-10 Å $\leq z \leq$ 10 Å) of the double-bond-deleted profiles contain contributions from only methyl and methylene groups.

1991). The transbilayer double-bond distribution, determined from neutron diffraction of DOPC specifically deuterated at the double bonds, is described by a pair of Gaussian functions each situated 7.88 ± 0.09 Å from the bilayer center with 1/e-halfwidths of 4.29 ± 0.16 Å (Wiener et al., 1991). Scaling the composition space representation of the double-bonds by the appropriate scattering length, maps the distribution to x-ray or neutron scattering-length space (Wiener and White, 1991*b*). The differing contributions of the double bonds to the absolute x-ray and neutron profiles is apparent. Subtraction of the double-bonds from each profile yields a region in the center of the bilayer (-10 Å $\leq z \leq$ 10 Å) that contains only methyls and methylenes as shown by the solid lines.

As given by Eq. 1, each of the scattering density profiles $\rho_X^*(z)$ and $\rho_N^*(z)$ can be expressed in terms of the composition space methyl and methylene distributions, $n_{CH3}(z)$ and $n_{CH2}(z)$, respectively. Inverting the two independent expressions of Eq. 1 yields $n_{CH3}(z)$ and $n_{CH2}(z)$ which are shown in Fig. 2 *A*. The squares represent the methyl (*lower set*) and methylene (*upper set*) distributions. Comparison of the two distributions provides evidence for substantial mixing of the acyl chains in the bilayer center. The shaded bands represent the experimental uncertainties of the distributions calculated by a Monte Carlo procedure (Press et al., 1989; Wiener and White, 1991*b*, *c*) using the uncertainties of the absolute-scale neutron and x-ray structure factors (Wiener and White, 1991*a*, *c*). The use of absolute scales removes all adjustable parameters from the analysis and thereby places stringent demands on the accuracy of the data. The data of Fig. 2 *A* show two imperfections which we attribute to inaccuracies in the higher order neutron structure factors which are at the limits of experimental precision. One is an apparent positive offset of the baseline for the CH_3 distribution and the other the ripples seen at $\sim \pm 6$ Å. The correct baseline can be established by fitting a Gaussian function with constant offset to the CH_3 distribution recognizing that the area under the Gaussian must be constrained to have an area of 4.00 which is the number of terminal methyls in the unit cell. A least-squares minimization fit yields a Gaussian with 1/e-halfwidth of 2.95 ± 0.28 Å offset from zero by 0.073 ± 0.038. The ripples at ±6 Å fluctuate around the offset and represent a form of Fourier noise due to slight distortions in the shape of the neutron profile arising from inaccuracies in the higher order neutron structure factors. Because the reduced χ^2 of the fit to the observed profile is <1 and the offset is within the $\pm 2\sigma$ domain of the experimental uncertainty, there is no statistical basis for rejecting the hypothesis that the fundamental shape of the terminal methyl distribution is Gaussian.

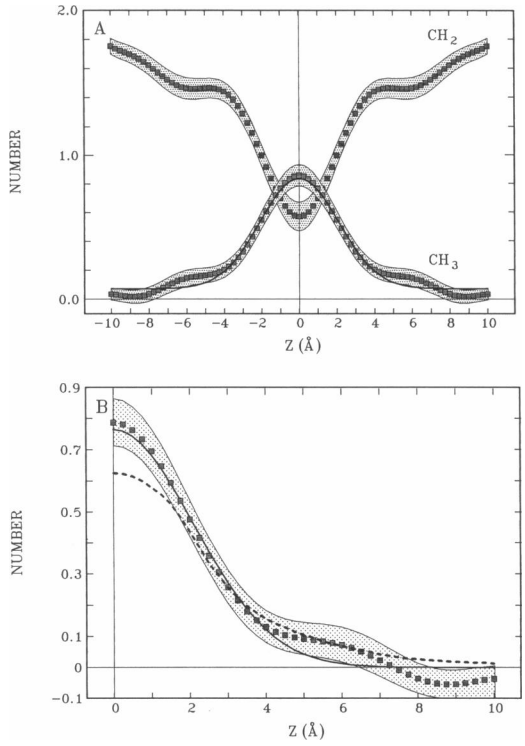

FIGURE 2 (*A*) Transbilayer distributions of methyls and methylenes in the interior of L_α-phase DOPC bilayers (66% RH, 23°C). The distributions are in the composition-space representation (Wiener and White, 1991*b*) (see Fig. 1). The squares represent the methyl (*lower set*) and methylene (*upper set*) distributions obtained from inversion of Eq. 1. The shaded bands represent the experimental uncertainties of the distributions calculated by Monte Carlo simulation (Press et al., 1989; Wiener and White, 1991*b*,*c*) using the experimental uncertainties of the scaled neutron and x-ray structure factors. The solid line represents the best Gaussian function fitted by least-squares minimization to the terminal methyl composition-space distribution. The Gaussian is located at the bilayer center and has an area of 4.00 (the number of methyls) with a 1/e-halfwidth of 2.95 ± 0.28 Å. The small offset, 0.073 ± 0.038, and the ripples at ±6 Å are probably due to inaccuracies in the higher order neutron structure factors. (*B*) Comparisons of the observed Gaussian representation of the terminal methyl distribution (*solid line*) and a theoretical distribution (*dashed line*) determined by Gruen and de Lacey (1984) with the observed CH_3 distribution. The offset of 0.073 has been subtracted from the observed distribution and its Gaussian representation. While the shape of the theoretical curve is different from the Gaussian curve, its full-width at half-maximum (FWHM) of 5.2 Å compares favorably with the 5.0 Å FWHM of the observed distribution. Possible causes of the differences in shape are discussed in the text.

Fig. 2 *B* compares the experimental data with our observed Gaussian representation of the terminal methyl distribution (*solid line*) and with a theoretical distribution (*dashed line*) obtained by Gruen and de Lacey (1984) by means of exhaustive simulation of

L_α-phase acyl chain conformations of a single twelve-carbon chain in a mean-field potential (Marcelja, 1974). Perfect agreement of the experimental and theoretical data cannot be expected because the simulation was performed at an equivalent area/lipid of ~ 65 Å^2 while our experiment was performed at ~ 60 Å^2. Also, the double bonds of DOPC may further confound the comparison. While the shape of the theoretical curve is different from the experimental curve, its full width at half maximum (FWHM) of 5.2 Å compares favorably with the 5.0 Å FWHM of the observed distribution. The most noticeable discrepancy between the Gaussian representation of the observed distribution and the theoretical distribution is the long tail of the latter. We note that a recent combined molecular and stochastic dynamics simulation of a seven-molecule DPPC monolayer (de Loof et al., 1991) at an equivalent area/lipid of 69 Å^2 yielded a terminal methyl distribution similar to that obtained by Gruen and de Lacey (1984). It remains to be seen whether or not this tail will appear in the experimental data at higher hydrations (i.e., larger areas/lipid).

For bulk liquid alkanes at 20–40°C and 1 atm, the molecular volume of a methyl group (V_{CH3}) is generally assumed to be 2.0–2.1 times that of a methylene group (V_{CH2}) (Reiss-Husson and Luzzati, 1964; Nagle and Wiener, 1988). Small (1986) has suggested, however, that a ratio closer to 1.25 is more appropriate. Although the wide-angle x-ray diffraction patterns of bulk alkanes and L_α-phase lipid bilayers are similar (Luzzati, 1968), acyl chains in bilayers have highly anisotropic motions (Seelig, 1977) and are subject to substantial van der Waals and hydration forces (McIntosh and Simon, 1986; Rand and Parsegian, 1989) at reduced water content. The possibility of methyl and methylene volumes that differ from those of bulk alkanes must be considered and our data permit this question to be addressed directly. Furthermore, our data permit a direct examination of Small's (1986) proposal. The bilayer profiles shown in Fig. 1 are on an absolute scattering length per unit length scale (Wiener and White, 1991*a*) so that the methylene and methyl distributions of Fig. 2 *A* represent the number of groups per unit cell per unit length, i.e., the composition of a unit cell is projected onto a line normal to the bilayer plane. We emphasize that these numbers result from the absolute scaling of the data and contain no adjustable parameters. If the average cross-sectional area S of the unit cell is known, then the volume composition can be determined from

$$S = V_{CH3} \cdot n_{CH3}(z) + V_{CH2} \cdot n_{CH2}(z) + V_{C=C} \cdot n_{C=C}(z), \quad (2)$$

recalling that the units of $n(z)$ are number/length. Near the center of the bilayer, where the contribution of the double-bonds is minimal,

$$S \approx V_{CH3} \cdot n_{CH3}(z) + V_{CH2} \cdot n_{CH2}(z). \quad (3)$$

If the volume of either the methyl or methylene is known, then the other can be estimated at $z = 0$ because $n_{CH3}(0)$ and $n_{CH2}(0)$ are now known.

The volumes of methyls and methylenes in alkanes can be determined by plotting alkane volume as a function of the number of methylene groups. The slope and intercept provide values of methylene and methyl volumes, respectively, and this analysis has been applied to data from liquid alkanes and fluid phospholipids at atmospheric pressure (Nagle and Wiener, 1988). Here we extend the method to include volumetric data from alkanes over a range of hydrostatic pressures (Dymond et al., 1979; Dymond et al., 1982) to examine the possible effect of any putative effective pressure (hydration or other) being applied to the bilayer under the conditions of reduced hydration described here. As shown in Fig. 3, the methylene volume is nearly invariant, $V_{CH2} \approx 27$ Å^3, between 1 atm and 2,000 atm whereas V_{CH3} decreases quite drastically from 54 Å^3 ($V_{CH3}/V_{CH2} = 2.0$) to 42 Å^3 ($V_{CH3}/V_{CH2} = 1.6$). We therefore made the reasonable assumption that $V_{CH2} \approx 27$ Å^3 in fluid bilayers and calculated V_{CH3} with the prediction that any pressure effects upon packing would be manifested as a decrease in methyl volume. We note that Small (1986) suggests

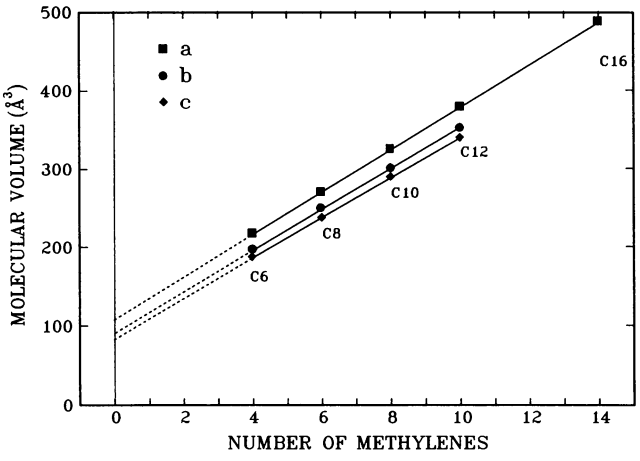

FIGURE 3 Plots of the molecular volumes of *n*-hexane (C6), *n*-octane (C8), *n*-decane (C10), *n*-dodecane (C12), and *n*-hexadecane (C16) versus number of methylenes at several hydrostatic pressures based upon the volumetric data obtained by Dymond et al. (1979, 1982). Curves *a*, *b*, and *c* are for pressures of 1 atm, 1,000 atm, and 2,000 atm respectively. The slopes of the curves yield the volume per methylene and the intercepts the total methyl volumes at each pressure. The slopes of the curves are (*a*) 27.1 ± 0.1 Å^3, (*b*) 26.0 ± 0.1 Å^3, and (*c*) 25.7 ± 0.2 Å^3 per methylene. The intercepts (total methyl volumes) are (*a*) $2 \cdot 54.3 \pm 0.2$ Å^3, (*b*) $2 \cdot 47.0 \pm 0.1$ Å^3, and (*c*) $2 \cdot 42.3 \pm 0.4$ Å^3.

this type of analysis is best performed using a reduced-temperature scale defined relative to the melting point of each alkane. This would have the effect in Fig. 3 of increasing the slopes and decreasing the intercepts of the curves. Small (1986) has thus suggested that $V_{CH2} \approx 29$ Å^3 and $V_{CH3} \approx 36$ Å^3 at 1 atm.

We determined the absolute specific volume of multilamellar dispersions of DOPC in excess water to be 0.9925 ± 0.0005 ml/g using the method of Wiener et al. (1988). With 5.36 ± 0.08 waters per DOPC at 66% RH (White et al., 1987), S is calculated in the manner of Nagle and Wiener (1988) to be 59.3 ± 0.7 Å^2. Assuming $V_{CH2} = 27 \pm 1$ Å^3 (Fig. 3) and using our values of $n_{CH3}(0)$ and $n_{CH2}(0)$ (Fig. 2), Eq. 3 yields $V_{CH3} = 57.2 \pm 3.6$ Å^3 where the uncertainty includes the errors in the absolute neutron and x-ray data comprising the Fourier density profiles (Fig. 1). If we use Small's (1986) result that $V_{CH2} = 29$ Å^3 and $V_{CH3} = 36$ Å^3, Eq. 3 yields $S = 45$ Å^2 which is grossly different than our measured area. Even if one assumes that the offset of 0.073 in Fig. 2A was removed inappropriately, the area/lipid would be 48 Å^2. Areas as small as these are observed only for sub-gel and gel phase lipids (Nagle and Wiener, 1988). Because calorimetric and wide-angle x-ray measurements indicate that DOPC at 66% RH is in a fluid state (unpublished observations), Small's (1986) methylene and methyl volumes appear to be inconsistent with our experimental results.

We conclude that the generally accepted values of 27 and 54 Å^3 are correct near the center of the bilayer. Assuming these values are the correct ones, the volume contribution of the double-bonds near the bilayer center can be estimated from Eq. 2. In the region between $z = \pm 2$ Å, we find $V_{C=C}$ is about 46 Å^3 which compares favorably with the value of 43 Å^3 determined by Requena and Haydon (1975) for n-alkenes. Calculations of volumes outside of $z = \pm 2$ Å are problematic because of the Fourier ripples in the CH_2 and CH_3 distributions (Fig. 2A) so we do not know if the methyl, methylene, and double-bond volumes are constant throughout the hydrocarbon core. However, the analysis of the complete structure of L_α-phase DOPC is consistent, within experimental uncertainty, with the assumption that the generally accepted values are accurate throughout the bilayer (Wiener and White, 1992). Overall, our results indicate that, despite the highly anisotropic motion of the acyl chains compared to bulk alkanes, the molecular packing of the chains in liquid-crystalline bilayers is equivalent to that of liquid alkanes as commonly assumed (Tardieu et al., 1973; Nagle and Wiener, 1988). Further, the large hydration and van der Waals "pressures" in the bilayer have no apparent effect upon packing.

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