Area/lipid of bilayers from NMR

J. F. Nagle
Departments of Biological Sciences and Physics, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213 USA

ABSTRACT Values of area per lipid A ranging from 56 to 72 Å^2 have been reported from essentially the same S_{CD} data from DPPC in the L_α phase. The differences are due primarily to three separate binary choices in interpretation. It is argued that one particular combination is best; this yields $A = 62 \pm 2$ Å^2 for DPPC at 50°C. Each preceding interpretation agrees with at least one of the three present choices and disagrees with at least one.

INTRODUCTION

Although lipids in biomembranes undergo many fluctuations and shape changes, it is of general interest to obtain reasonably accurate measurements of the average structure of the membrane, such as the thickness of the hydrophobic barrier and the area A per lipid at the aqueous interface. One particular use for A is in computer simulations, such as Monte Carlo (1) or molecular dynamics (2); such simulations usually require choosing A in advance. Simulated properties, such as the ratio of trans to gauche rotations or the NMR order parameters, depend sensitively upon both A as well as upon the model Hamiltonian, making evaluation of the success of simulations uncertain.

Measurements of A have frequently been reported using the so-called Luzzati method (3) which involves weighing out known amounts of water and lipid and determining the lamellar spacing using x-ray diffraction. This method is, at best, inaccurate, as seen by the results for the benchmark system, fully hydrated DPPC lipid bilayers in the L_α phase, where A ranges from 57.6 to 70.9 Å^2 (4) at 50°C. At worst, this method involves systematic errors because defect regions in multilamellar vesicles probably contain a disproportionate amount of water, but do not contribute to the lamellar D spacing (5), thereby overestimating the value of A, as recently shown for $L_{\beta'}$ phase lecithins (6).

Nuclear magnetic resonance has also been used to obtain A for lipids, with reported values including 58.6 Å^2 (7), 56 Å^2 (8), 69 Å^2 (9), and 71.7 Å^2 (10), covering a range of uncertainty that is nearly identical with the range obtained from x-ray studies. Unlike the x-ray data, however, the NMR data used to obtain this range are basically identical. The basic data consist of carbon-deuterium order parameters S_{CD} for temperatures between the main transition and 50°C. All studies obtain values in the range -0.20 ± 0.02 for S_{CD} for carbons $n = 4$–8 in the plateau region, and these experimental uncertainties do not account for the large range in the reported values of A. Rather, this is due to differences in other data that were employed with the nmr data and in the theories used to obtain A.

The purpose of this paper is to discuss whether it is possible to obtain a reasonably accurate value of A from S_{CD} data and to estimate the probable errors. This involves a careful discussion of the assumptions and models used, as well as some estimates of quantities that have not been measured. An alternative, purely empirical, approach has recently been advocated (11), but this approach relies upon the uncertainties in the x-ray determinations mentioned above. While there remains some basic uncertainty in the approach of this paper, it is small enough that useful new estimates of A can be made.

BASIC RELATIONS

Hydrocarbon chains are very nearly tetrahedral, as shown in Fig. 1 *a*. It has therefore been natural to place the chains on a diamond lattice (7, 10). A priori the single most likely orientation of the diamond lattice with respect to the bilayer is the one shown in Fig. 1 *a*, and this is the orientation that has been previously employed because a simple formula can be obtained that does not involve unknown parameters. It is clear, however, that a distribution of orientations should occur for each CD$_2$ group, as has been suggested by molecular dynamics simulations (2). Therefore, this section will allow for general angular rotation, which is most commonly described in terms of the three Euler angles, ϕ, θ, and ψ (12), but rotation around the first Euler angle ϕ does not affect any results so it is ignored. The sketch in Fig. 1 *b* shows how the other Euler angles rotate the axes. The price to be paid for this more realistic model is that the formula is not so simple, and it does involve unknown parameters. Nevertheless, this is an essential step in order to estimate the uncertainty in the result for A and to show that the result obtained from the conventional choice of orientation is a good approximation.

The goal of this section is to obtain a basic relation between the CD order parameter for the nth CD$_2$ group $\langle S_n \rangle$ and the mean distance $\langle d_n \rangle$ that the chain travels along the bilayer normal at this same C$_n$D$_2$ group. Both these quantities are obtained from the z-coordinates of the unit tetrahedral vectors shown in Fig. 1 *a:*

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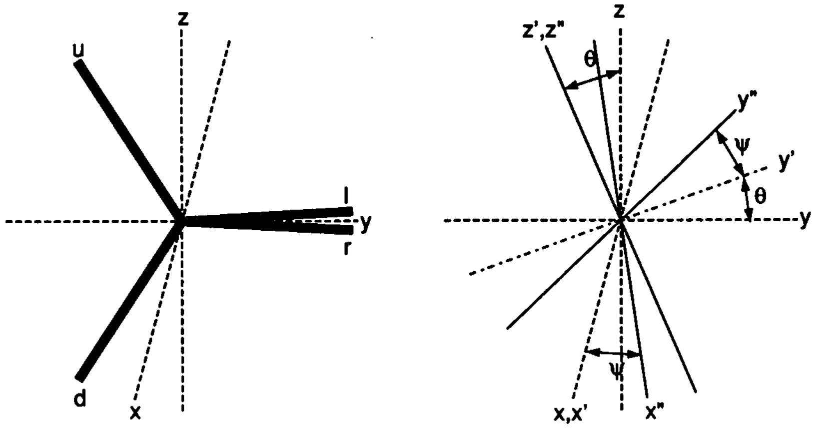

FIGURE 1 The left hand figure shows the special orientation for one site of a diamond lattice with bold lines in the directions *u*, *d*, *l*, and *r*. The normal to the bilayer is along the *z*-axis. The right hand side shows the Euler angle θ for tilting the diamond lattice away from the *z*-axis, with new axes x', y', z', and the Euler angle ψ for rotation of the lattice around the new z'-axis to the final x'', y'', z'' orientation.

$$\begin{aligned} z_u &= p\cos\theta - q\sin\theta\cos\psi \\ z_d &= -p\cos\theta - q\sin\theta\cos\psi \\ z_l &= -p\sin\theta\sin\psi + q\sin\theta\cos\psi \\ z_r &= p\sin\theta\sin\psi + q\sin\theta\cos\psi, \end{aligned} \tag{1}$$

where $p = (2/3)^{1/2}$ and $q = (1/3)^{1/2}$ and θ and ψ are the Euler angles indicated in Fig. 1 *b*.

There are clearly six possible orientations of a CD_2 group on the tetrahedron shown in Fig. 1 *a*, corresponding to the six ways to place two D atoms on the four bonds. Each orientation will occur with a certain probability; let us designate both the orientation and the probability as shown in Fig. 2. Because each CD_2 must have some orientation, the probabilities are normalized by

$$a + b_1 + b_2 + b_3 + b_4 + c = 1. \tag{2}$$

It is then straightforward, if tedious, to show that the order parameters S_i are given by

$$\begin{aligned} S_a &= (-\cos^2\theta + \sin^2\theta\sin^2\psi)/2 \\ S_c &= (+\cos^2\theta - \sin^2\theta\sin^2\psi)/2 \\ S_{b1} &= \sin\theta\cos\psi(+\cos\theta - \sin\theta\sin\psi)/2^{1/2} \\ S_{b2} &= \sin\theta\cos\psi(+\cos\theta + \sin\theta\sin\psi)/2^{1/2} \\ S_{b3} &= \sin\theta\cos\psi(-\cos\theta - \sin\theta\sin\psi)/2^{1/2} \\ S_{b4} &= \sin\theta\cos\psi(-\cos\theta + \sin\theta\sin\psi)/2^{1/2}. \end{aligned} \tag{3}$$

Notice that Eq. 3 gives the same value for S_{bi} for i = 1–4 when $\theta = 0 = \psi$, and this is the reason for grouping these four orientations together. For general values of θ and ψ it is tempting to try to reduce the number of cases using symmetry, but there is no general symmetry. For example, even though the probability of $-\psi$ is the same as $+\psi$, the probabilities b_1 and b_2 will be exchanged upon changing the sign of ψ. Summing the products of the order parameters with the probabilities of the particular orientations of the CD_2 group yields the average order parameter, $\langle S\rangle$

$$\begin{aligned} \langle S\rangle = {} & [(c-a)/2](\cos^2\theta - \sin^2\theta\sin^2\psi) \\ & + [(b_1 + b_2 - b_3 - b_4)/2^{1/2}]\sin\theta\cos\theta\cos\psi \\ & + [(b_2 + b_4 - b_1 - b_3)/2^{1/2}]\sin^2\theta\sin\psi\cos\psi. \end{aligned} \tag{4}$$

Next, we consider the distance *d* traveled along the bilayer normal. Clearly, for each placement of the deuteriums the chain can travel in two directions, with the distance traveled for one direction being the negative of the distance traveled for the other. Previous studies have assumed that only one of these directions has non-zero

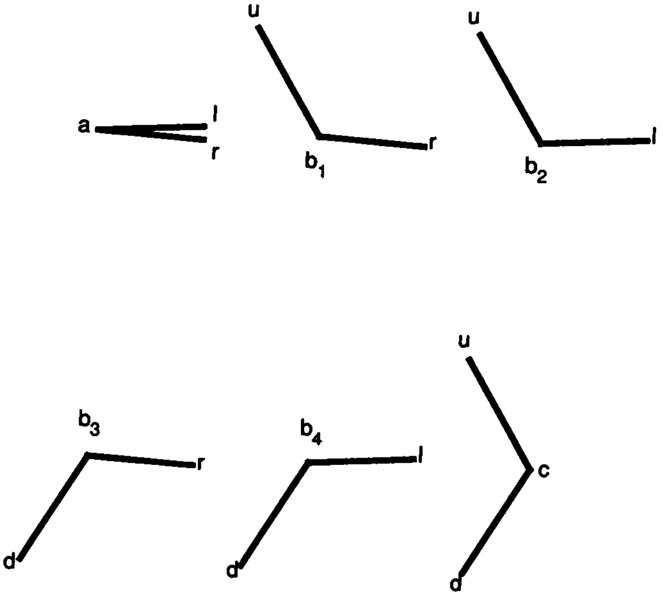

FIGURE 2 The six sets of directions for the two CD bonds are labelled a, b_1, b_2, b_3, b_4, and c. The directions *u*, *d*, *l*, and *r* are the same as shown in Fig. 1 *a* and this drawing is for $\theta = 0 = \psi$.

probability, and that there is zero probability for an upturn, where a chain reverses direction along the bilayer normal (9, 10, 13, 14). Let us defer discussion of this assumption of no upturns to the next section. Using it, one obtains for the distances traveled along the bilayer normal

$$\begin{aligned} d_a &= 2p\cos\theta \\ d_c &= 2p\sin\theta\sin|\psi| \\ d_{b1} &= p(\cos\theta - \sin\theta\sin\psi) - 2q\sin\theta\cos\psi \\ d_{b2} &= p(\cos\theta + \sin\theta\sin\psi) - 2q\sin\theta\cos\psi \\ d_{b3} &= p(\cos\theta + \sin\theta\sin\psi) + 2q\sin\theta\cos\psi \\ d_{b4} &= p(\cos\theta - \sin\theta\sin\psi) + 2q\sin\theta\cos\psi, \end{aligned} \tag{5}$$

where p and q are the same as in Eq. 1. Summing the products of the distances with the probabilities of the particular orientations of the CD_2 groups yields the average distance $\langle d \rangle$ per methylene group along the bilayer normal

$$\begin{aligned} \langle d \rangle / 1.27\ \text{Å} = {} & [a + (b_1 + b_2 + b_3 + b_4)/2]\cos\theta \\ & + c\sin\theta\sin|\psi| \\ & + [(b_3 + b_4 - b_1 - b_2)/2^{1/2}]\sin\theta\cos\psi \\ & + [(b_2 + b_3 - b_1 - b_4)/2^{1/2}]\sin\theta\sin\psi, \end{aligned} \tag{6}$$

where 1.27 Å is the total undirected distance covered by actual (slightly non-tetrahedral) CD_2 groups (15).

Even with the assumption that there are no upturns, there is no simple relation between $\langle d \rangle$ and $\langle S \rangle$. However, the following exact relation will still be useful:

$$\begin{aligned} \langle d \rangle / 1.27\ \text{Å} = {} & (^1/_2)\cos\theta - \langle S \rangle / \cos\theta \\ & + [(a - c)/2]\sin\theta\tan\theta\sin^2\psi \\ & + c\sin\theta\sin|\psi| \\ & + [(b_2 + b_3 - b_1 - b_4)/2]\sin\theta\sin\psi \\ & - [(b_1 + b_3 - b_2 - b_4)/2^{1/2}] \\ & \times \sin\theta\tan\theta\sin\psi\cos\psi. \end{aligned} \tag{7}$$

When θ is set equal to zero, this somewhat daunting expression simplifies considerably to the expression usually employed (7, 10, 13, 16):

$$\langle d \rangle / 1.27\ \text{Å} = (1 - 2\langle S \rangle)/2, \tag{8}$$

which allows one to determine $\langle d \rangle$ just from the experimental CD_2 order parameters $\langle S \rangle$. It may also be noted that Seelig and Seelig (14) used a slightly different formula from Eq. 8, but that this was corrected in the appendix to (7).

TABLE 1 **Approximate dependence of $\langle d \rangle$/1.27 Å upon θ and ψ for $\langle S \rangle$ = −0.2 and $a - c$ = 0.4**

$\langle \sin^2\psi \rangle$ θ	0	0.25	0.50	0.75	1.0
0°	0.700	0.700	0.700	0.700	0.700
15°	0.690	0.693	0.697	0.700	0.704
30°	0.664	0.678	0.693	0.707	0.722

We will now argue that the simple expression in Eq. 8 is a fairly good approximation to the full expression in Eq. 7 for values of $\langle S \rangle$ near −0.20. First, it may be noted that θ can be restricted to values in the range from 0 to 35.3°, because further rotations in θ are identical to lesser rotations with the different configurations in Fig. 2 interchanged. The first two terms on the right hand side of Eq. 7 are quantitatively rather similar to Eq. 8, though the sum is smaller. The factor $(a - c)/2$ in the third term can be estimated from the special case $\theta = 0$, for which $a - c = -2\langle S \rangle = 0.4$, so a is at least as large as 0.4. Since c-type orientations move in the unfavorable direction roughly perpendicular to the bilayer normal, it is most likely that $b_i > c$. Since $a + c + \sum b_i = 1$, the average b_i would be ~0.1–0.14 and c would be ~0.1–0.02. Therefore, the fourth term in Eq. 7 is less than half as large as the third term. We also note that, if very small upturns were allowed, associated with taking the opposite direction for c-type orientations, then the fourth term would be even smaller. As θ and ψ are varied from zero, the degeneracy in the b_i is split. By Eq. 5 the lengths d_{b1} and d_{b4} change oppositely to d_{b2} and d_{b3}, respectively, so if $b_1 < b_2$, then $b_4 < b_3$. Therefore, the last term in Eq. 7 is likely to be small because of the coefficient in square brackets. Since d_{b2} and d_{b3} increase as ψ increases from zero, while d_{b1} and d_{b4} decrease, and since the b_i for longer d_i should be favored, the fifth term on the right hand side of Eq. 7 should be positive, though not as large as the third and fourth terms due to the pairwise subtraction of the b_i. It would therefore seem that one may reasonably neglect the last three terms in Eq. 7. Doing so leads to the results shown in Table 1. A priori $\langle \sin^2\psi \rangle$ might be expected to be ½. Molecular dynamics simulations (2) suggest that $\langle \sin^2\psi \rangle$ is less than ½, but not near 0, of course. The average value of $|\theta|$ will certainly be smaller than 30° since θ is centered around 0, and 15° seems a generously large estimate. This suggests that the most appropriate range in Table 1 is 0.693–0.697. Since the neglected terms in Eq. 7 are positive, it seems that the simple Eq. 8 is likely to be accurate to within a percent. It may be emphasized, however, that if simulations were run at appropriate values of A and if statistics were obtained for all the unknown terms in Eq. 7, then one could use Eq. 7 to lead, in an iterated fashion, to better values of A. In the meantime, we will use the simpler Eq. 8.

It is essential to work with a reasonably accurate representation of hydrocarbon chains, as will now be demonstrated by considering an alternative formula due to De Young and Dill (9). These authors prefer a vastly simplified model that forces the hydrocarbon chains onto a simple cubic lattice, and they assume that the lattice has zero tilt θ so all bonds are either parallel or perpendicular

TABLE 2 **Upturn statistics* for $A = 62$ Å^2**

Carbon number n	<8	9	10	11	12	13	14
% Upturned	0	1.8	2.4	7.1	7.7	11.9	13.7

* Data provided by H. L. Scott (personal communication).

to the bilayer normal. Their parameter σ in their Eq. 8 is the same as our $\langle d\rangle/1.27$ Å. They also use $S_{\text{mol}} = -2S_{\text{CD}}$ so their Eq. 8 can be rewritten as

$$\langle d\rangle/1.27\ \text{Å} = (1 - 4\langle S\rangle)/3. \qquad (9)$$

For $\langle S\rangle = -0.2$, this yields a value of 0.6 for $\langle d\rangle/1.27$ Å, which differs by more than 15% from the more accurate value given by Eq. 8 obtained using tetrahedral geometry.

NO UPTURN ASSUMPTION

The major assumption untested in the preceding section was that there are no upturns, i.e., reversals along the bilayer normal, for the hydrocarbon chains. This is a standard assumption in all the preceding interpretations of S_{CD} data. One obvious reason is that the formula in the preceding section becomes even more complex and indeterminate if upturns are included. A priori, it is a reasonable assumption that there are few upturns because an upturn leads to packing difficulties. A chain with an upturn requires at least twice as much area at the level of the upturn, and if it turns back down again, it requires at least three times as much area. This creates congestion for neighboring chains, which can only be relieved by a large decrease in their entropy, thereby decreasing the probability of such an event.

Xu and Cafiso (17) have detected a close proximity of some terminal methyls with the membrane interface, indicating that up-turns do exist. They were not able, however, to quantitate the percentage, mentioning only that a few percent might suffice to account for their data. It may also be noted that their data were for sonicated unilamellar vesicles which are more disordered than multilamellar vesicles and would be expected to have more upturns.

Although it is impossible to determine the amount of upturning from any current measurements, this is an issue that can be addressed from simulations performed at the appropriate areas/molecule. Current Monte Carlo simulations performed by H. L. Scott give roughly the statistics for DMPC shown in Table 2. Not surprisingly, the number of upturns increases towards the terminal methyl end of the chain, with percentages that are large enough that one would wish to modify the formula in the preceding section for $n > 10$. We will not make this modification, but let it be noted that upturns will decrease $\langle d\rangle$ by roughly twice their percentage. More importantly, it appears that the standard assumption of few upturns is valid for $n < 8$, which is in the plateau region for the S_{CD} order parameter.

AN INTERESTING FALLACY

As was first noted by Seelig and Seelig (14), the mean length $\langle L\rangle$ of the hydrocarbon chains can be obtained from formulae such as Eq. 8 by summing $\langle d(n)\rangle$ over all carbon numbers n up to the end N of the chain. The formula for $\langle L\rangle$ has been refined by Salmon et al. (13) to take account the terminal methyl and inequivalences in the two hydrocarbon chains.

Schindler and Seelig (7) used their value of $\langle L\rangle$ to obtain A from the volume V

$$A\langle L\rangle = V, \qquad (10)$$

and V was estimated using gel phase values. Of course, the volume of the hydrocarbon chains in the L_α phase is larger than in the gel phase, so Thurmond et al. (10) used subsequent volume measurements (18, 19) with their refined formula for $\langle L\rangle$ to obtain new and larger values of A. (An additional minor refinement would be to include an extra length for the terminal methyl in the formula for $\langle L\rangle$ because it has an extra volume ($V_{\text{CH3}} = 2V_{\text{CH2}}$) which is included (10) in the calculation of V.)

This approach to obtaining A is elegantly simple. However, Eq. 10 is incorrect! The easiest way to show this is with a simple counterexample. Fig. 3 shows one specific conformational state of the hydrocarbon region of a bilayer. This particular conformational state is not meant to represent a model of the bilayer. It is clearly too simple, with only two different chain conformations and with sharp edges between only two regions, from 0 to L_1 and from L_1 to L_2. Nevertheless, this simple model embodies an essential idea. The total volume per chain V is just the average area, A, multiplied by the length to the midplane, so

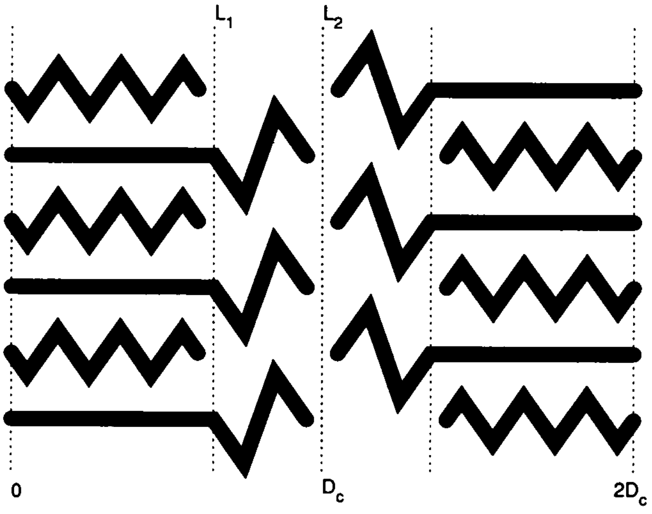

FIGURE 3 A caricature showing how it is possible that the hydrocarbon chain volume V can be greater than $A\langle L\rangle$. The hydrocarbon chains are sketched as bold dark lines and the bilayer normal is horizontal. The number of carbons in each chain is the same, but the more disordered chains have total length L_1 compared to L_2 for the longer chains that are more ordered near the headgroups.

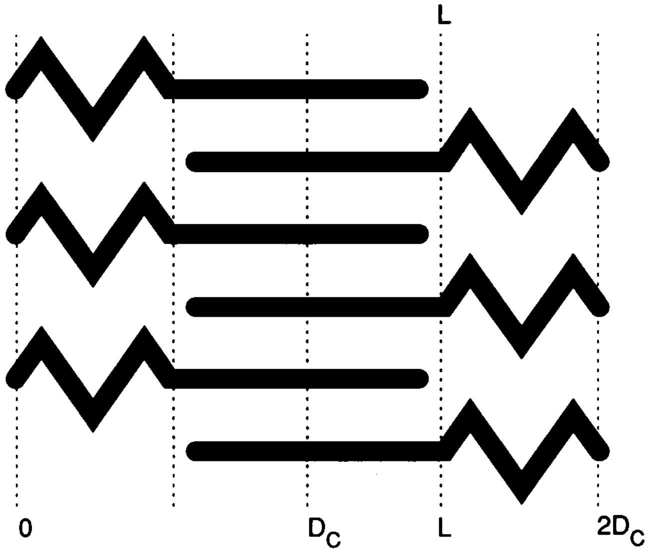

FIGURE 4 A caricature showing how it is possible that the hydrocarbon chain volume V can be less than $A\langle L\rangle$ if there is interdigitation of the terminal methyl ends of the chains. The density of packing of chains then requires these ends to be more ordered, contrary to the S_{CD} data.

$$V = AL_2. \tag{11}$$

However, the average length of a chain is

$$\langle L\rangle = (L_1 + L_2)/2 \tag{12}$$

which is smaller than L_2. Therefore, there is a contradiction with Eq. 10. For this particular conformation, Eq. 10 overestimates A.

Fig. 4 shows a specific conformational state where Eq. 10 underestimates A because in this case $\langle L\rangle$ is larger than the thickness of half the bilayer, $D_c/2$. Which kind of deviation from Eq. 10 prevails for the wide range of conformations in a typical bilayer? The answer is clear when the order parameters as a function of carbon position n are compared to S_{CD} data. For the conformation shown in Fig. 3 there is a plateau in S_{CD} for the half of the chain closest to the headgroup, $1 < n < N/2$. For the terminal methyl half of the chain, $N/2 < n < N$, there is another plateau, but corresponding to values of S_{CD} closer to zero since the longer chains with length L_2 are much more disordered near the terminal methyl ends. In contrast, for the conformation shown in Fig. 4 the plateau for $N/2 < n < N$ has an order parameter that is further from zero than for $1 < n < N/2$. Since the actual S_{CD} data approach zero as n approaches the terminal methyl end N, the type of conformation shown in Fig. 3 prevails, not the type shown in Fig. 4.

RESULTS AND DISCUSSION

A better way to use the S_{CD} order parameters to calculate A will now be illustrated. First, Eq. 8 will be used to calculate $\langle d\rangle$. Second, to avoid the problem of upturns, only $\langle d\rangle$ for carbons $n < 9$ in the plateau region will be used. The area will be calculated using

$$A = V_{CH2}/\langle d\rangle. \tag{13}$$

Use of $V_{CH2} = 27.6$ Å^3 for DPPC at 50°C (4, 18) and $S_{CD} = -0.20$ yields $\langle d\rangle = 0.889$ Å and $A = 62.1$ Å^2. Uncertainties are estimated at ±2 Å^2 due to estimated uncertainties of 1% in V_{CH2}, of 1% in Eq. 8, and of 5% in the values of the experimental plateau S_{CD} order parameters at 50°C (9, 13, 14).

The use of values of S_{CD} only in the plateau region follows from an early discussion of deGennes (20). DeGennes emphasized that the conservation of flow of hydrocarbon chains from the headgroup interface requires a constant or plateau value of the order parameter up to a value n_c. Methylenes with $n < n_c$ are located at distances z from the headgroup where no chain terminations (terminal methyls) occur. If there are no upturns in this region, then each plane perpendicular to the bilayer normal must cross every chain just once and Eq. 13 follows. In contrast, methylenes with $n > n_c$ occupy space beyond the ends of some of the terminal methyls and chain termination requires that the remaining longer chains become more disordered to fill in the area vacated by the terminating chains as shown in Fig. 3. The exclusive use of plateau values of S_{CD} has been employed by De Young and Dill (9) and it was mentioned by Thurmond et al. (10) as an alternative way to obtain A. Here it is argued that it is by far the better way.

Previous papers differed methodologically from the present one in at least one of three ways. The first way was the use of gel phase volumes (7, 9) which ignore the considerable volume expansion and thereby underestimate A. The second way was the use of a simple cubic geometry (9) instead of Eq. 8; this overestimates A. The third was the use of average chain lengths $\langle L\rangle$ and Eq. 10 (7, 10, 16) instead of plateau values and Eq. 13; this also overestimates A.

Finally, the new estimate for A is the basis for new estimates for other structural quantities. In particular, the number of water molecules n_w per lipid between bilayers in multilamellar vesicles is given by

$$n_w = (AD/2 - V_L)/V_w, \tag{14}$$

where, for fully hydrated DPPC at 50°C, the lamellar repeat spacing D is 67.0 Å, the lipid volume V_L is 1,232 Å^2 and the partial specific volume of a water molecule is 30.3 Å^3 (4). From the present result for A, Eq. 14 yields $n_w = 28.0$. This is intermediate between previous estimates of 23.0 and 37.7 from x-ray diffraction (see (4)). Also, previous analysis (4) has obtained a hydrocarbon volume V_C of 884 Å^3. Together with the present value of A this yields 28.5 Å for the average hydrocarbon thickness $2D_C$ of the DPPC bilayer at 50°C. Since the D spacing is usually measured to be ~67 Å, this leaves 38.5 Å for the combined length of the headgroup and the water along the bilayer normal. If each headgroup has an average length D'_H along the bilayer normal of 8 Å (4), this in turn leaves 22.5 Å for the water space D_W between bilayers in multilamellar DPPC vesicles at 50°C. This space contains only $2(n_W - n'_W) = 46$ water molecules, the remaining $n'_W = 5$ water molecules per lipid being mixed with the headgroups in the headgroup space.

The author thanks Michael Brown for careful reading and criticism of the manuscript and H. L. Scott for providing the simulation data shown in Table 2.

This research was supported by grant GM-44976 from the United States National Institutes of Health.

Received for publication 4 November 1992 and in final form 7 January 1993.

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