Hexane Dissolved in Dioleoyllecithin Bilayers Has a Partial Molar Volume of Approximately Zero†

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Received July 26, 1984; Revised Manuscript Received November 30, 1984

ABSTRACT: Neutron diffraction has been used to measure the amount and distribution of hexane incorporated from the vapor phase into oriented dioleoylphosphatidylcholine bilayers at 66% relative humidity. We reported earlier that hexane at low concentrations is located largely in a zone 10 Å wide at the center of the bilayer [White, S. H., King, G. I., & Cain, J. E. (1981) *Nature (London) 290*, 161–163]. Extending these studies to high hexane concentrations, we find no readily apparent change in the volume of the hydrocarbon region of the bilayer even though more than one hexane molecule per lipid enters the region. The hexane partial molar volume in the bilayer hydrocarbon region is thus approximately zero. Within our statistical confidence limits, the partial molar volume is certainly no greater than one-third the molecular volume of the hexane. Further, analysis of the data suggests that the mass density of the bilayer is considerably less than 1 in the absence of hexane. These findings are in conflict with the assumptions usually made about lipid bilayers and their interaction with nonpolar hydrophobic molecules. In the course of these experiments, we found that standard methods of interpreting diffraction results were not suitable for our purposes. We thus developed several new methods which are summarized in the text and two appendixes. One of these methods allows us to define with precision the width of the hydrocarbon core of the bilayer. The other provides a means of calculating the effects of changes in the absolute scaling of the bilayers with changes in composition without placing the structures on an absolute scattering length density scale.

The organizing structural element of biological membranes is the lipid bilayer which is generally viewed as a quasi-two-dimensional "solvent" for hydrophobic protein "solutes" (Singer & Nicolson, 1972). The details of the protein interaction with the bilayer are central to understanding the architecture of membranes and the mechanisms for insertion and transport of proteins into and across membranes. There are two issues fundamental to understanding these interactions: (1) the nature of the bilayer as a solvent for hydrophobic molecules and (2) the nature of peptide–bilayer interactions at the level of the peptide bonds and the amino acid side groups. The work reported here is concerned with the first issue which we and others have studied for a number of years through examination of the interactions of alkanes with bilayers (White, 1976, 1977, 1980; Simon et al., 1979; McIntosh et al., 1980; White et al., 1981; Kita et al., 1981; Kita & Miller, 1982). The molecular packing arrangement of the alkanes in the bilayer and structural changes in the bilayer induced by the interactions are fundamental problems most easily addressed by using neutron diffraction techniques. This paper focuses on these problems.

The results of our previous neutron diffraction studies (White et al., 1981) on hexane at low concentrations showed that the amount of hexane going predominantly into the central 10-Å region of the bilayer was an approximately linear function of hexane vapor pressure, that the bilayer structure was not changed by the presence of the hexane, and that the *d* spacing of the multilayers did not change over this range. We have now extended our measurements to include higher hexane vapor pressures. The results are quite unusual and have forced us to examine carefully the assumptions one generally makes about the mixing of alkanes with bilayers.

Several assumptions are customarily adopted regarding the bilayer and its interaction with alkanes. In the absence of alkanes, fully hydrated bilayers generally have a mass density of about 1 (Reiss-Husson, 1967; Huang & Charlton, 1971; Nagle & Wilkinson, 1978; Knoll, 1981). Lacking data to the contrary, one generally assumes the density at lower relative humidities is also about 1 [e.g., see Parsegian et al. (1979)]. The packing of the acyl chains in bilayers is assumed to be the same as for bulk alkyl liquids (Reiss-Husson & Luzzati, 1964) because of the 4.6-Å diffracted intensity observed for both alkane liquids and bilayers (Levine & Wilkins, 1971). With these "usual" assumptions and knowledge of the amount of water present in dioleoylphosphatidylcholine (DOPC)[1] bilayers at 66% relative humidity (six H_2O molecules per lipid) (Jendrasiak & Hasty, 1974), and the *d* spacing of the system (49.7 ± 0.5 Å), one can calculate the area per lipid in the interface (S_0) to be 60.8 ± 1.2 Å^2 and the thickness of the hydrocarbon region (D_h) to be 32.3 ± 0.6 Å in the hexane-free bilayer.

On the basis of measurements of black lipid films (White, 1976, 1977; Andrews et al., 1970; Requena & Haydon, 1975), we have until now assumed the following concerning the mixing of alkanes with bilayers: (1) The area per lipid molecule in the bilayer is independent of the amount of alkane in the bilayer. (2) Alkane mixes in an ideal volumetric way; i.e., the partial molar volume of the alkane is the same as the molecular volume in bulk. (3) The alkane is concentrated in the center of the bilayer. The last assumption has been confirmed for DOPC multilayers at moderate relative humidities (White et al., 1981). The first two assumptions lead to the

† Parts of this research were carried out at the Brookhaven National Laboratory under the auspices of the U.S. Department of Energy with the additional support of the National Science Foundation. This research was supported by grants from the National Institutes of Health and National Science Foundation. Parts of this work were presented in preliminary form at the Brookhaven Symposium in Biology, May 31–June 4, 1982.

[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

0006-2960/85/0424-4637$01.50/0

conclusion that D_h should increase by 7.4 Å if one hexane per lipid enters the bilayer.

We have been examining these important assumptions for DOPC multilayers using neutron diffraction and direct measurements of the uptake of radiolabeled hexane. The results reported in this paper show that, for DOPC multilayers at 66% relative humidity, D_h does not increase when hexane is incorporated into the bilayer and that the area per molecule is probably larger than expected. These results lead to the following conclusions: (1) Hexane enters the bilayer while the volume of the hydrocarbon region remains constant within experimental error; i.e., the partial molar volume of the hexane is approximately zero. (2) The mass density of the bilayer is apparently smaller than 1.

To arrive at these conclusions, we had to develop two new analytical methods. One of them uses strip-function models to define with excellent precision of the width of the hydrocarbon core of the bilayer. Knowledge of this width is essential for understanding how hexane, or any other molecule for that matter, packs into the bilayer. The other method arose because it is necessary to account for shifts of the structures of the absolute scattering length density scale resulting from changes in composition *without* knowing the true absolute scale of the structure. It was possible to solve this problem because we determined the composition of the system independently of the diffraction experiments. Both methods are described briefly under Discussion and in Appendixes A and B. They will be described more fully in other publications later.

Materials and Methods

Materials. DOPC was purchased from Avanti Polar-Lipids, Inc. (Birmingham, AL), hexane (pesticide grade) from Burdick and Jackson Laboratories, Inc. (Muskegon, MI), perdeuterated hexane from KOR Isotopes, Inc. (Cambridge, MA), and ^{14}C-labeled DOPC from New England Nuclear (Boston, MA). Tritiated hexane was synthesized from 1-bromohexane via reduction with $LiAl^2H_4$. All alkanes used were checked for purity by using gas chromatography and found to be greater than 99% pure. Thin-layer chromatography was used to check lipid purity.

Sample Preparation. DOPC multilayers were deposited on a quartz slide by evaporation from a 1:1 chloroform–methanol solution and mounted in the neutron beam in a sealed and thermostated aluminum chamber containing reservoirs of a saturated salt solution and a hexane–hexadecane mixture. A saturated solution of sodium nitrite in water was used to maintain the relative humidity at 66% (T = 22.5 °C). The vapor pressure, P, of the hexane was controlled by a hexane–hexadecane mixture which behaves nearly ideally (McGlashan & Williamson, 1961) so that $P = X_M P_0$ where P_0 is the vapor pressure of pure hexane and X_M is the mole fraction of hexane in the mixture.

Direct Uptake Experiments. For these measurements, a chamber similar to the diffraction chamber was constructed. Bilayers were formed on microscope slide coverslips from ^{14}C-labeled DOPC. The uptake of ^{3}H-labeled hexane was determined under conditions chosen to ensure equilibrium and to minimize the loss of hexane to the atmosphere during transfer of the coverslips to the scintillation fluid.

Neutron Diffraction Experiments. The neutron diffraction data were collected on either the H-4 satellite or the H-4 beampipe at the high-flux beam reactor at Brookhaven National Laboratory. For the satellite experiments, a two-dimensional position-sensitive detector was used to collect the data, and a step-scanning diffractometer with a single gas-filled proportional counter was used in the θ–2θ mode on the beampipe. Since the orientation of the samples was very high (mosaic spread of about 0.1°, full width at half-maximum), it was necessary to rotate the sample in θ to obtain the complete pattern. Thus, it was necessary to correct for the variation of the sample–beam intersection with θ. In addition, an inverse Lorentz correction factor of h, where h is the diffraction order, was used.

Analysis of Diffraction Data. The methods used for analyzing and interpreting the data are described under Discussion and in Appendixes A and B. The calculations require that the scattering lengths (B) of the water (B_W), hexane (B_H), and DOPC (B_L) be known. These are arrived at by summing up the known individual scattering lengths of each atom in a molecule. A list of these is given in Schoenborn (1975). The values we used are $B_W = -0.18 \times 10^{-12}$ cm, $B_H = -1.36 \times 10^{-12}$ cm, and $B_L = 3.21 \times 10^{-12}$ cm.

Results

The signs of the structure factors were obtained by first determining the native DOPC (no hexane) phases. This was accomplished by utilizing H_2O–D_2O exchange (Blasie et al., 1975; Worcester & Franks, 1976; Franks & Lieb, 1979) and the swelling theory (Worcester & Franks, 1976; Franks & Lieb, 1979; Worthington et al., 1973; King, 1971). The signs of orders for h = 1–4 were determined to be –, –, +, –, which are the same as those determined by others for dipalmitoylphosphatidylcholine (DPPC) (Zaccai et al., 1975), DPPC–cholesterol (Blasie et al., 1975), and egg lecithin–cholesterol (Worcester & Franks, 1976).

Figure 1a shows the effects on the structure factors of increasing X_M up to 0.8. For values of X_M beyond 0.8, the diffraction pattern begins to disappear, suggesting a major phase change in the system. When X_M is returned to zero for the sample, the bilayer structure reappears, indicating the reversible nature of the phase change. We do not know the structure of this new phase. Over the entire range X_M = 0–0.8, the d spacing remains constant at 49.7 Å with a precision of ±0.5 Å for both protonated and deuterated lipid. The structure factors remain essentially constant (within a precision of ±2%) up to X_M = 0.4, change between X_M = 0.4 and 0.5, and then remain essentially constant up to the highest hexane vapor pressures giving rise to measurable diffraction patterns. There is clearly a major structural change in the transition region (see Table I). The constant d spacing for all X_M and the lack of dependence of the structure factors on X_M outside the transition region lead to the conclusion that the partial molar volume of hexane is zero except at the transition (see Discussion). Figure 1b shows that when D-hexane is employed, there is a significant variation of the structure factors of the multilayers with X_M. The resulting structures (Figure 2) clearly show the presence of D-hexane. Therefore, at both high and low hexane concentrations, significant amounts of hexane are being incorporated into the bilayer.

Figure 2 shows the structures derived from the structure factors shown in Figure 1. The changes in bilayer structure above and below the transition at X_M = 0.4–0.5 can be seen by comparing curves A and B. Note the decrease in headgroup scattering density and the slight narrowing and smoothing of the hydrocarbon region. Curve A represents the structure of the bilayer (independent of X_M) for $X_M \leq 0.4$ and curve B the structure for $X_M \geq 0.5$. Curve C demonstrates dramatically the presence of the deuterated hexane. Subtraction of curve B from curve C yields the distribution of hexane across the bilayer [see White et al. (1981)].

Figure 3 shows the amount of hexane incorporated into DOPC multilayers as a function of hexane partial pressure

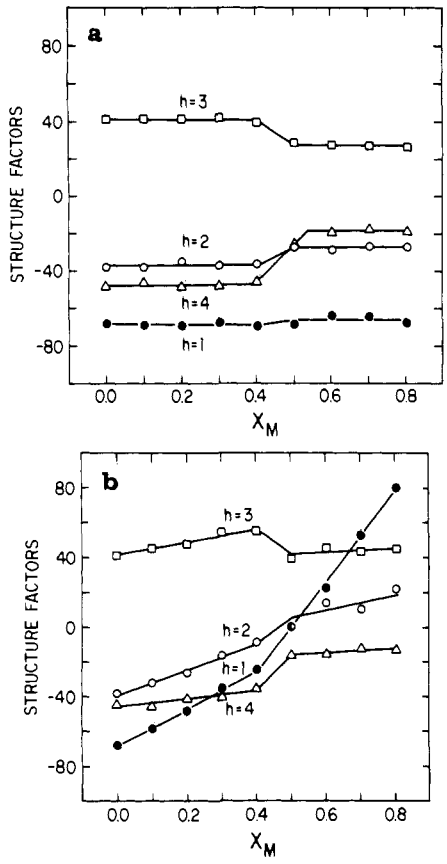

FIGURE 1: Neutron structure factors (a and b) for oriented DOPC multilayers containing various amounts of protonated or deuterated *n*-hexane. The structure factors (for h = 1–4) of bilayers in the presence of protonated hexane (a) and deuterated hexane (b) are plotted here as a function of X_M which is the mole fraction of hexane in the hexane–hexadecane mixture used to set the hexane vapor pressure in the sample chamber. The diameters of the data points indicated the precision of the measurements. The actual amounts of hexane in the bilayers are shown in Figure 2 and Table I. The d spacing remains constant at 49.7 ± 0.5 Å for all values of X_M for both deuterated and protonated hexane.

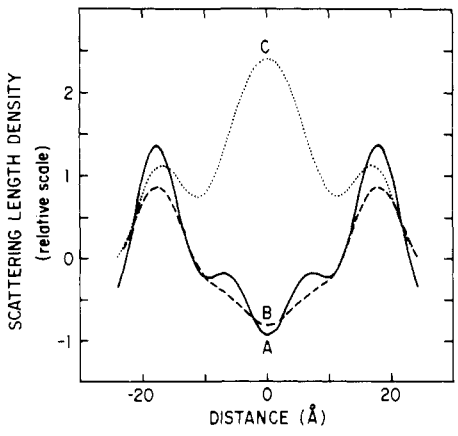

FIGURE 2: Neutron scattering density profiles of DOPC bilayers on a relative scale. Shown here are structures for DOPC bilayers (T = 22.5 °C, 66% relative humidity) in the absence of hexane (curve A, solid line), bilayers containing protonated *n*-hexane (curve B, dashed line; X_M = 0.8), and bilayers containing deuterated *n*-hexane (curve C, dotted line; X_M = 0.8).

determined by using [^{3}H]hexane. The increased slope of the curve in Figure 3 at high hexane partial pressures implies that the incremental increase in D-hexane uptake increases significantly upon going from low to high hexane partial pressures. We have measured the water content of the bilayers

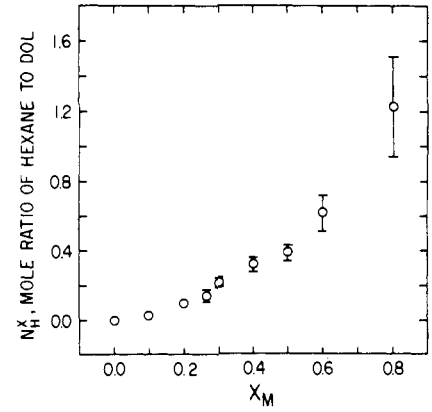

FIGURE 3: Uptake of hexane by DOPC multilayers at 22.5 °C, 66% relative humidity. The amount of hexane in the bilayer is given as the mole ratio (N_H^X) of hexane to phospholipid. The mole fraction of hexane in the bilayer is $X_B = N_H^X/(1 + N_H^X)$. X_M is the mole fraction of hexane in the hexane–hexadecane mixture used to maintain the vapor pressure of hexane in the chamber (see Figure 1).

as a function of X_M (unpublished results). Our results are in agreement with those of Jendrasiak & Hasty (1974) for X_M = 0. We find a small decrease as X_M is increased, but the change is negligible for these calculations. We assume throughout that there are six waters per lipid.

Discussion

The structure factors (Figure 1) provide a means for testing the usual assumption of ideal volumetric mixing of alkanes with the hydrocarbon region of the lipid bilayer. We calculate structure factor variations with X_m for three extreme cases of bilayer volume changes with hexane incorporation. In *case I*, we consider ideal volumetric mixing such that the entire volume change is due to an increased area per lipid (S_X). In *case II*, we again assume ideal mixing, but now we assume that the entire volume change is due to an increased hydrocarbon thickness (D_h). In *case III*, we assume highly *nonideal* behavior; we make the counterintuitive assumption that neither D_h nor S_X changes with hexane entry. That is, in *case III* we assume the partial molar volume of hexane in the hydrocarbon core is zero so that the volume of the core remains constant. Structure factors for these three cases are calculated and compared with those observed by finding strip-function models (vide infra and Appendix B) whose Fourier transforms (i.e., structure factors) agree with the observed structure factors at X_M = 0 and with those extrapolated back to X_M = 0.5 from X_M = 0.6–0.8. (The reason for this extrapolation is that the observed data for X_M = 0.5 are in the "transition region".) The parameters of these strip-function models were then changed accordingly for each case and the resulting structure factors calculated.

The dashed lines in Figure 4 shows the variation in the structure factors with X_M for *case I*, the dotted lines the variation for *case II*, and the solid lines the variation for *case III*. The *case II* curve is very far away from the data points, and it is safe to conclude that the thickness of the hydrocarbon is not increasing. We thus rule out *case II*. *Cases I* and *III* are more difficult to distinguish with this type of analysis without extensive statistical arguments. Rather than deal with statistics which provide little physical insight, we examine relative changes in bilayer structure as hexane enters the bilayer. *Case I* assumes constant hydrocarbon core mass density while *case III* assumes constant hydrocarbon core volume. Thus, the central question is the following: "How much does the volume of the hydrocarbon core change with the entry of hexane?"

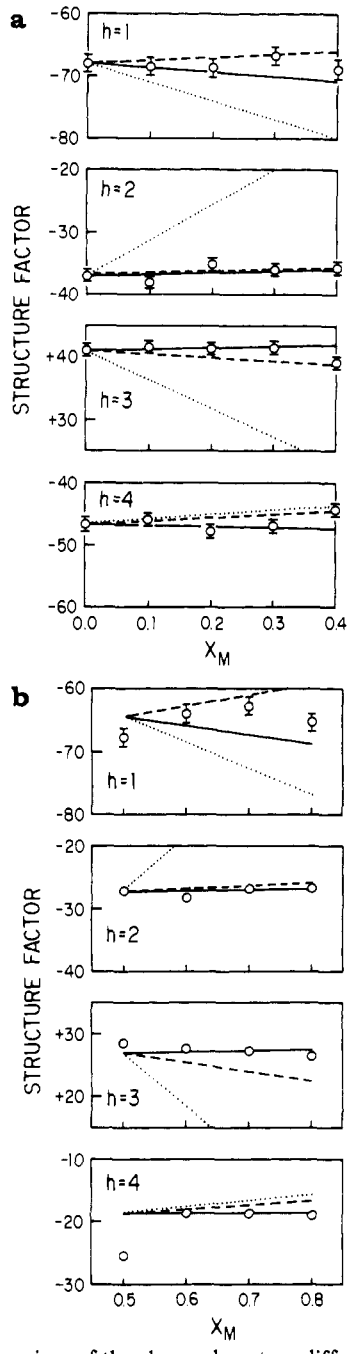

FIGURE 4: Comparison of the observed neutron diffraction structure factors from DOPC vs. X_M with those expected in three extreme cases (see Discussion). The error bars or the diameters of the data points indicate the standard deviation of the measurements. In *case I* (dashed line), we consider the variations of structure factors vs. X_M that are calculated if ideal volumetric mixing of the hexane with the lipid were occurring entirely through increases in area per lipid (S_X). In *case II* (dotted line), we show the variations in structure factors vs. X_M that are calculated if ideal volumetric mixing were occurring entirely through increases in hydrocarbon thickness (D_h). *Case III* (solid line) shows the variation in structure factors vs. X_M calculated if mixing of the hexane with the lipid were occurring with no changes in either D_h or S_X (i.e., hexane partial molar volume of zero).

To examine this question, one needs to know the *relative* changes in S_X and D_h with X_M. The usual structural analysis (Luzzati, 1968; Rand & Luzzati, 1968) requires a knowledge of the mass density of the hydrated bilayer or at least an assumption about the partial molar volumes of the lipid and water at the particular hydration chosen. Generally speaking, the densities of the water and lipid are assumed to be unity. One can then calculate the area per lipid (S_0), the thickness of a water layer, and the thickness of the lipid layer (D_l) which is simply the d spacing minus the water layer thickness. We did not wish to make the assumptions required by such an analysis, and besides, the analysis does not permit one to estimate the thickness of the hydrocarbon core. We therefore used a different approach which is described in the following paragraphs.

The ratio of the lipid area (S_X) at a particular X_M to the lipid area (S_0) of the hexane-free bilayer can be obtained from eq 1 which equates the volume integral of the scattering length density of the bilayer (derived from diffraction data) with the scattering length of the bilayer calculated from the composition of the system (measured independently) and the known scattering lengths of the components:

$$(1/K)\left[\int_0^{D/2} \rho_{BL}(z)\, dz\right]_X S_X = B_L + N_W{}^X B_W + N_H{}^X B_H \quad (1)$$

where

$$\rho_{BL}(z) = (2/D)\sum_h F(h) \cos(2\pi hz/D) + C_X \quad (2)$$

In eq 1, $N_W{}^X$ and $N_H{}^X$ are, respectively, the numbers of water and hexane molecules per lipid; B_L, B_W, and B_H are the scattering lengths of the lipid, water, and protonated hexane, respectively. C_X is a constant for each X_M. The constant $1/K$ is the unknown absolute scaling factor (instrumental constant). A more complete description of the relation between relative and absolute scattering length densities and the meaning of C_X is included in Appendix A. There are three unknowns in these equations: $1/K$, S_X, and C_X. S_X is not really a problem because we are interested only in *relative* changes which can be found by comparing evaluations of eq 1 at different values of $N_H{}^X$. Thus, the main problem is to eliminate C_X and $1/K$ from the equations. This can be done by using a self-consistency requirement if D_h (the thickness of the hydrocarbon core) is known.

Considering only the hydrocarbon region, one can write

$$(1/K)\left[\int_0^{D_h/2} \rho_{BL}(z)\, dz\right]_X S_X = 2B_{ac} + N_H{}^X B_H \quad (3)$$

where B_{ac} is the scattering length (-1.32×10^{-12} cm) of the acyl chain (C-2 through C-18). This equation can be solved for $(1/K)S_X$ to eliminate it from eq 1. The result is an equation containing two integrals to be evaluated from the known structure factors [$F(h)$] and two unknowns (C_X and D_h). The latter enters the equation through the limits of integration in eq 3. We describe below how D_h can be determined from strip-function models of the bilayers. These models are also important for the evaluation of the integrals in eq 1 and 3. The problem with their evaluation is that we have only 4 orders of diffraction data which introduce termination artifacts via eq 2. The strip-function models allow us, in effect, to average out these artifacts. Given a value of D_h and a rational way of evaluating the integrals, values of C_X can be determined from eq 1–3 combined. Relative changes in S_X can be subsequently determined from eq 1 and 2 at different values of $N_H{}^X$.

Strip-function models (Figures 5 and 6; Appendix B) are best considered as *averages* of relative scattering length density across the appropriate layers of the bilayer such as the head-group region, the methylene region, and the central methyl trough region. The thicknesses and relative scattering length densities of these layers can be determined by varying them as parameters until the structure factors (h = 1–4) of the model fit the observed structure factors within experimental error. The derivation of appropriate strip-function models is

Table I: Structural Parameters of Dioleoyllecithin Multilayers (T = 22.5 °C, 66% Relative Humidity) Containing Various Amounts of *n*-Hexane[a]

X_M	X_B	D_l (Å) (±1.0 Å)	D_h (Å) (±0.6 Å)	S_X/S_0 (±0.04)	V_{hc}/V_0 (±0.05)	V_{hc}^*/V_0	d_{hc}/d_0
0.0	0.00	44.2	28.2	1.00	1.00	1.00	1.00
0.1	0.03	44.2	28.2	1.00	1.00	1.01	1.01
0.2	0.10	44.2	28.2	0.99	0.99	1.03	1.04
0.3	0.18	44.2	28.2	1.03	1.03	1.05	1.02
0.4	0.24	44.2	28.2	1.02	1.02	1.07	1.05
0.5	0.28	46.2	27.0	1.08	1.03	1.10	1.05
0.6	0.38	46.2	26.0	1.16	1.07	1.14	1.06
0.7	(0.49)	46.2	26.0	1.18	1.09	1.21	1.10
0.8	0.55	46.2	26.0	1.16	1.07	1.28	1.17

[a] X_M is a measure of the hexane vapor pressure surrounding the bilayers (see Materials and Methods). X_B is the mole fraction of hexane in the bilayer measured by using radiolabeled hexane (see Figure 3). The d spacing (D) is 49.7 ± 0.5 Å, independent of X_M. As determined from the strip-function models (Appendix B), D_l is the lipid layer thickness including head groups and D_h the thickness of the hydrocarbon layer. D_l is derived by the method of Luzzati (1968) and D_h as outlined in Appendix B. S_X is the area per lipid molecule, and $V_{hc} = S_X D_h/4$. $S_X = S_0$ and $V_{hc} = V_0$ at $X = 0$. V_{hc} represents the volume of the hydrocarbon layer (including hexane) per acyl chain. V_{hc}^*, shown for comparison, is the volume expected if the hexane is mixed with the acyl chains in an ideal volumetric way. d_{hc} is the estimated mass density of the hydrocarbon core with $d_{hc} = d_0$ when $X_M = 0$. By use of the approach of Reiss-Husson (1967), V_0 is estimated to be 475 Å^3 and d_0 = 0.83 g/cm^3. Using the value of D_h given in the table and the estimated value of V_0, we find S_0 = 67.5 ± 1.4 Å^2. Making the usual assumptions (see text), we expect S_0 to be 60 Å^2, D_h to be 32 Å in the absence of hexane, and D_h to increase by about 7 Å at the highest hexane concentration.

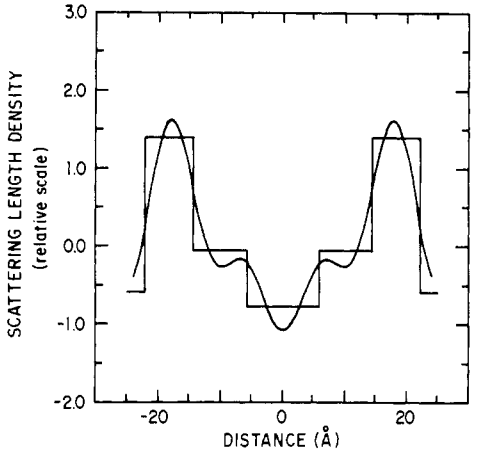

FIGURE 5: Comparison of neutron scattering length density Fourier synthesis and strip-function model representation of hexane-free DOPC bilayer. The parameters of the strip-function model were obtained by requiring a best fit to the observed neutron structure factors (see Discussion and Appendix B).

described in more detail in Appendix B.

The derived value for the hydrocarbon thickness (D_h) is the most important parameter obtained from the models. Indeed, it is pivotal from both the conceptual and analytical points of view. Conceptually, we are considering how the hexane molecules affect the hydrocarbon volume which is determined by D_h and the area per phospholipid (S_X). In the simplest case where S_X is fixed, changes in the hydrocarbon thickness reveal directly the changes in volume. More generally, one must also expect changes in S_X which we can now evaluate as described above. Analytically, a proper value of D_h is critical for the evaluation of eq 3. We note that our results are relatively insensitive to some of the strip-function parameters such as the shape of the methyl trough. On the other hand, they are extraordinarily sensitive to changes in D_h which is the most crucial parameter. It was for precisely this reason that *case II* (ideal mixing at constant S_X) could be easily discounted by using the approach summarized in Figure 4.

A good fit of the strip models to the observed structure factors *cannot* be obtained with D_h values significantly different than the best-fit values. In the absence of hexane, our strip model results (Table I) give D_h = 28.2 ± 0.6 Å. Lewis & Engelman (1983) obtained a value of 27 ± 1 Å by using a totally independent method and somewhat different experimental conditions (vide infra). We have made direct measurements of D_h in our system using DOPC deuterated at the C-2 positions of the acyl chains (unpublished results). The center to center spacing of the C-2 carbons on opposite sides of the bilayers is 28 ± 1 Å—completely consistent with our value calculated from the strip models.

Strip-function models determined as described above were thus used in the evaluation of eq 1–3. Table I lists the important molecular ratios at the various hexane concentrations. In all cases, V_{hc} is the hydrocarbon region volume per lipid acyl chain and thus includes both the acyl chain and hexane volumes. V_{hc} is constant (within the experimental uncertainty of 5%) over the whole range of hexane concentrations, although there appears to be a small volume increase in going from X_M = 0.4 to X_M = 0.5. This is true despite the fact that at X_M = 0.8 the bilayer is more than an equimolar mixture of alkane in lipid. Thus, we are led to the conclusion that the partial molar volume of hexane in the hydrocarbon region of the bilayer is very small and not different from zero within our experimental error. We cannot rule out the possibility of a small partial molar volume for hexane in the hydrocarbon core, but it is unlikely to be larger than one-third the molar volume of pure hexane based upon our experimental error. Despite its counterintuitive nature, we are forced to believe that *case III* explains most accurately our experimental results. One of the conclusions of Table I is that above the transition, D_h should decrease.

When the *whole* system consisting of lipid, water, and hexane is considered, the partial molar volume of the hexane is constant to within experimental error *except* for the transition region between X_M = 0.4 and X_M = 0.5. The structural data in Table show a significant overall expansion of the system volume through the transition. The appropriate measure of the system volume is $(DS_X)/2$ which shows a 15% increase. The partial molar volume of hexane, in the transition, can be estimated to have a minimum value of approximately 700 Å^3, which is far greater than the molar value of 217 Å^3 for the pure liquid. There is clearly a major structural change at the transition. Its origin is unknown, but because the volume of the hydrocarbon region does not change by more than 5%, the major effect must involve the water and polar group. There must be a compensating volume increase in this region which offsets the lack of one in the hydrocarbon region even though hexane never enters the polar region. This seems to be the case. The volume of the polar region can be calculated for Table I by using the equation $V = S_X(D - D_h)/2$. Assuming S_0 = 65 Å^2 (*vide infra*), the volume increases by 235 Å^3 when

X_M is changed from 0 to 0.8. This is approximately the volume of a hexane molecule, and we note that at $X_M = 0.8$ about one hexane per phospholipid enters the bilayer. This result is *not* built into our models or analysis explicitly in any way. It comes out of the analysis solely as a result of fitting the strip models to the diffraction data.

Further examination of Table I reveals another result which may at first seem somewhat surprising. As the bilayer passes through the transition between $X_M = 0.4$ and 0.5, the hydrocarbon thickness (D_h) *decreases*, the "fluid space" between the head group ($D - D_l$) *decreases*, and the "head-group" thickness ($D_l - D_h$) *increases*. Preliminary measurements of changes in the transbilayer spacing of deuterium attached at the double bonds of DOPC are consistent with decreases in D_h at high hexane concentrations (White et al., 1984). We note in Appendix B that the strip models are of little value for examining fine structure in the head-group region. Nevertheless, these seemingly contradictory results can be reasonably explained. The head-group thickness becomes wider, but the area per molecule has increased as well. Not shown in the table is the fact that the scattering length density of the head group has decreased concomitantly so that the total scattering length (the area under the head-group strip) tends to be preserved. This can be seen from the structures in Figure 2 (compare curves A and B). We suggest that the width of the head group, is increasing as a result of a major reorganization of the head groups and water which involves a simultaneous increase in area and some sort of reorientation. We must emphasize, though, that we do not have adequate resolution in this region to separate the individual contributions of the head groups and water. We only claim that changes must be occurring which we do not yet understand in detail.

Up to this point, the analysis has dealt with results on a *relative* scale so that only *ratios* such ad N_W^X, N_H^X, S_X/S_0, and V_{hc}/V_0 have been determined. This is a result of not knowing the area per lipid (S_0) in the hexane-free case (see Appendix A). From a determination of the mass density of the multilamellar DOPC–H_2O system, the value of S_0 could be obtained. These measurements are currently in progress. We can nevertheless test the result of making the usual assumption that the mass density of the bilayer system is 1. If we take $S_0 = 60$ Å^2, which results from this assumption, we can utilize our result of essentially constant hydrocarbon volume to examine its implications regarding the mass density of the hydrocarbon region. In the absence of hexane, $d_{hc} = d_0$ is calculated to be 0.93 g/cm^3 given $D_h = 28.2$ Å (Table I). When $X_M = 0.8$, d_{hc} is calculated to be 1.14 g/cm^3, assuming $D_h = 26.0$ Å and taking $S_X = 60(28.2/26) = 65$ Å^2 (recognizing that the hydrocarbon volume remains constant). For comparison, we note that 9-octadecene in the liquid state has a density of 0.85 g/cm^3 (Weast, 1965) and in the crystalline state a value of about 1.0 g/cm^3 (Privalko, 1975). Hence, the usual assumption of $S_0 = 60$ Å^2 leads to unreasonable hydrocarbon mass densities when $X_M = 0.8$.

We can obtain estimates of S_0 in several ways. Our results imply little change in hydrocarbon volume over the whole hexane range. Assuming that the hydrocarbon mass density cannot exceed that of crystalline hydrocarbons (about 1 g/mL), we take d_{hc} as 1.0 g/cm^3 for $X_M = 0.8$. From Table I, $d_{hc}/d_0 = 1.17$, suggesting that $d_0 = 0.85$ g/cm^3. The mass of an acyl chain (excluding the carbonyl group) is 3.93×10^{-22} g, giving a chain volume of 462 Å^3. With $D_h = 28.2$ Å (Table I), we find $S_0 = 65$ Å^2. The other approach, noted in Table I, is to assume the hydrocarbon region has the density expected of bulk alkyl liquids. This yields a value of 67.4 ± 1.4 Å^2. We thus estimate that S_0 is 5–7 Å^2 greater than expected from the usual assumptions. This conclusion has important implications regarding the overall mass density of the system as described in the next paragraph. In passing, we note that the acyl chain volume of 475 Å^3 (Table I) expected from bulk alkyl liquids is very close to the value of 462 Å^3 calculated by assuming $d_{hc} = 1.0$ for $X_M = 0.8$. This suggests that the usual assumption of bulk alkyl densities for the hydrocarbon region in the absence of hexane is probably correct.

Lewis & Engelman (1983) report $D_h = 27 \pm 1$ Å and $S_0 = 70.1 \pm 2.7$ Å^2 for DOPC vesicles in excess water. These numbers agree with those reported above and in Table I. We calculate from our d spacing and the composition of the system that the mass density of the hydrated bilayer system with no hexane lies between 0.80 and 0.86 g/mL (allowing S_0 to range between 65 and 70 Å^2). This density is significantly less than the value of about 1.02 g/mL measured for egg lecithin vesicles in excess water (Huang & Charlton, 1971). Lewis & Engelman (1983) did not report density values for the DOPC vesicles, but the values were apparently greater than 1 because the vesicles could be pelleted by centrifugation. Because our hydrocarbon region structural parameters are in agreement with theirs, the packing arrangement of the head groups and water in oriented multilayers at low relative humidities must be considerably different from vesicles in excess water.

Finally, we discuss some of the possible thermodynamic implications of our results. We can estimate the free energy of transfer of hexane from the pure liquid to the bilayer employing the data in Figure 2 and Table I. For low values of X_M, the free energy of transfer is about 300 cal mol^{-1} [see White et al. (1981) for details of the calculations]. For the higher values of X_M (greater than 0.5), this drops to about 200 cal mol^{-1}. These numbers are considerably smaller than those reported by Simon et al. (1977) for DOPC in excess water. Nevertheless, their conclusions regarding the enthalpic and entropic contributions to the free energy of transfer of hexane to DOPC bilayers in excess water may still be generally valid in our case. They observe a large negative enthalpy (hexane prefers bilayer) offset by a large negative entropy (hexane relatively more ordered in bilayer). Application of these observations suggests that hexane would like to fill voids in the bilayer, but the ensuing tighter packing causes a compensating decrease in entropy. This entropy decrease should normally limit the solubility of the hexane in bilayers in excess water. In bulk alkyl systems, this entropy decrease prevents the entrance of hexane into the "free volume" of, e.g., hexadecane so that ideal volumetric mixing is the preferred state of affairs. Our results suggest that the packing density of the hexane in the hydrocarbon core far exceeds that expected in such bulk systems. A possible explanation is that there is a compensating increase in entropy due to the interaction of water and polar groups. Consistent with this hypothesis, we showed above that the volume (but not the mass) of the polar region increases at the transition by about the volume of a hexane molecule for each lipid. This reasoning may explain why our free energy of transfer is smaller than that of Simon et al. (1977) and why we find much larger amounts of hexane than they. If similar effects occur for the hydrophobic side chains of proteins in membrane lipids, this has potentially intriguing implications for the state of the lipids near the protein ("boundary lipids") and for protein insertion into and transport across membranes.

We do not know at this time how generally applicable our conclusions are to lipids other than DOPC or for other relative humidities. The results presented here do indicate that caution should be exercised in making the usual assumptions without

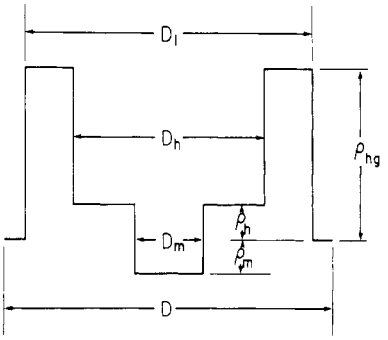

FIGURE 6: Structural parameters for strip-function models of bilayers. D_l is the lipid thickness determined for the hexane-free case by using the method of Luzzati (1968) and D_m the width of the methyl trough taken from White et al. (1981). The other parameters were varied simultaneously by computer until the Fourier transform of the model yielded Fourier coefficients (model structure factors) which agreed with the experimental structure factors to within experimental error. D is the d spacing (measured), ρ_{hg} the average scattering length density of the head-group region, ρ_h the average scattering length density of the hydrocarbon core near the head groups, and ρ_m the average scattering length density of the methyl trough region. The scattering length densities are on an arbitrary relative scale and are defined relative to what would be considered the water layer in the Luzzati (1968) treatment. See Appendix B for further details.

experimental verification. The work of Franks & Lieb (1979) on the uptake of general anesthetics by lecithin–cholesterol bilayers is of special interest in this context. The structural changes observed at high anesthetic concentrations were similar to ours *and* unaccompanied by changes in d spacing. This suggests that the effects we observe may be widespread. Dr. Keith Miller and his colleagues (Kita et al., 1981; Kita & Miller, 1982), as well as Franks & Lieb (1981), have measured the partial molar volumes of several compounds dissolved in bilayers and find them not too different from bulk molecular volumes. However, these compounds are considerably more water soluble than hexane and may be located more in the interfacial regions. Technical problems prevent direct thermodynamic measurements of partial molar volumes in our system.

In summary, we can say the following about the usual assumptions for our system: (1) At low relative humidities, the mass density of the bilayer may be considerably less than 1. (2) The packing of the acyl chains in the absence of hexane is about the same as for bulk alkyl liquids. (3) Excluding the transition region, the area per molecule is independent of the amount of hexane in the bilayer. (4) Hexane does not mix in an ideal volumetric way. Its partial molar volume is approximately zero (excluding the transition region). (5) The hexane is located largely in the center of the bilayer.

Acknowledgments

We thank Dr. Benno P. Schoenborn and his staff for their advice and encouragement. The excellent technical help of Elizabeth Thompson is gratefully acknowledged. The work benefited greatly from discussions with Drs. Jay Edelman and Tom McIntosh. We are pleased to acknowledge Professor J. Seelig's gift of DOPC deuterated at the C-2 position.

Appendix

(A) The purpose of this appendix is to describe more fully the concepts underlying the analysis presented under Discussion. The fundamentals of the diffraction analysis of membrane systems are described succinctly in a review by Franks & Levine (1981).

Most diffraction analyses are performed by using *relative* scales of scattering length density. While this is satisfactory for many purposes, the placement of the data on an *absolute* scattering length density scale is essential for describing the absolute mass distribution of a structure. The scattering length density of a structure on an absolute (abs) scale is given by

$$\rho_{abs}{}^X(z) = \bar{\rho}_{abs}{}^X + \frac{1}{K}\frac{2}{D}\sum_1^h F_{obsd}{}^X(h)\cos(2\pi hz/D) \qquad (A1)$$

In this equation, the superscript X is a reminder that the scattering length density depends upon composition, i.e., the concentration of hexane in the vapor phase defined by X_M. The coordinate normal to the plane of the bilayer is z. $1/K$ is an instrumental constant, and D is the d spacing of the structure. The structure factors are given by

$$F_{obsd}{}^X(h) = S(h)[I_{obsd}(h)]^{1/2} \qquad (A2)$$

where $S(h)$ represents the phase factors and $I_{obsd}(h)$ represents the corrected observed (obsd) diffraction intensities. For centrosymmetric structures, $S(h) = \pm 1$. The average absolute scattering length density of the structure is given by

$$\bar{\rho}_{abs}{}^X = B_W n_W + B_H n_H + B_L n_L \qquad (A3)$$

where B_W, B_H, and B_L are the scattering lengths of the water, protonated hexane, and lipid, respectively, and n_W, n_H, and n_L are the numbers of water, hexane, and lipid molecules per unit volume, respectively. This equation may be written

$$\bar{\rho}_{abs}{}^X = \frac{2}{S_X D}(N_W{}^X B_w + N_H{}^X B_H + B_L) \qquad (A4)$$

where N_W and N_H are the numbers of water and hexane molecules per lipid molecule, respectively. This equation shows that $\bar{\rho}_{abs}$ depends on the area per lipid molecule (S_X) which in turn depends upon the mass density, d_X:

$$d_X = \frac{2}{S_X D}(N_W{}^X M_W + N_H{}^X M_H + M_L) \qquad (A5)$$

where M_W, M_H, and M_L are the molecular masses of the water, hexane, and lipid molecules, respectively. Note that the summation in eq A1 begins with $h = 1$. The equation could have been written by starting with $h = 0$ and incorporating $\bar{\rho}_{abs}$ as the zero-order term. Using this approach, one writes

$$\rho_{abs}{}^X(z) = \frac{1}{K}\frac{1}{D}\sum_{-h}^{+h} F_{obsd}{}^X(h)\cos(2\pi hz/D) \qquad (A6)$$

where

$$\frac{1}{K}\frac{1}{D}F_{obsd}{}^X(0) = \bar{\rho}_{abs}{}^X \qquad (A7)$$

Most structural analyses are concerned with spatial relationships between components rather than mass density relations. The zero-order term and the instrumental constant $1/K$ are thus usually ignored, and the data are considered on a *relative* scale. Thus

$$\rho_{rel}{}^X(z) = \frac{2}{D}\sum_1^h F_{obsd}{}^X(h)\cos(2\pi hz/D) \qquad (A8)$$

The relation between the relative and absolute scattering length densities is found from eq A1 and A8 to be

$$\rho_{abs}{}^X(z) = \bar{\rho}_{abs}{}^X + \frac{1}{K}\rho_{rel}{}^X \qquad (A9)$$

An important point to note is that relative scale data provide *no* absolute measure of the mass density of the systems or the area per lipid molecule. Relative changes in these values can, however, be determined as described under Discussion.

We now relate these ideas and equations to the equations used under Discussion. Comparison of eq 2 with eq A1 shows that

$$\rho_{BL}^{X}(z) = K\rho_{abs}^{X}(z) \quad \text{(A10)}$$

and that

$$C_X = K\bar{\rho}_{abs}^{X} \quad \text{(A11)}$$

These equations show that for a system whose composition is variable, the structures on a relative scale are affected by changes in the absolute scattering length density. Thus, in the analysis under Discussion, the parameter C_X is introduced to account for this fact. In essence, C_X sets the average scattering length density of the structure on the *relative* scale. If one considers several structures with different compositions, the average scattering length density levels will be separated by amounts proportional to the differences between their C_X's.

Equation 1 can be derived easily by noting that

$$\bar{\rho}_{abs}^{X} = \frac{2}{D}\int_0^{D/2} \rho_{abs}^{X}(z)\, dz \quad \text{(A12)}$$

given that $\rho_{abs}^{X}(z) = \rho_{abs}^{X}(-z)$. From eq A10, A11, and A12, one can write

$$C_X = \frac{2}{D}\int_0^{D/2} \rho_{BL}^{X}(z)\, dz \quad \text{(A13)}$$

Combining eq A4 and A11, one finds

$$C_X = K\frac{2}{S_X D}(N_W^X B_W + N_H^X B_H + B_L) \quad \text{(A14)}$$

Eliminating C_X between eq A13 and A14 yields eq 1.

(B) We describe briefly in this appendix the parameters of the strip-function models used in calculating the results shown in Table I. Figure 6 shows a strip model and the parameters necessary to specify it. The D's are distances, and the ρ's are scattering length densities (arbitrary scale). The general procedure is to set up a computer program which calculates the Fourier transform of a strip model and compares the Fourier coefficients (structure factors for the strip model) with the experimental structure factors at each X_M. The parameters are varied by the program until agreement between the model and actual structure factors is achieved to within experimental error.

Excluding the d spacing (D) which is measured, there are six parameters. Because there are generally only 4 orders of diffraction data at each X_M, the problem is underspecified. We therefore made assumptions about two of the parameters, D_l and D_m. D_l was calculated initially from the zero hexane data by using the methods of Luzzati (1968) and Rand & Luzzati (1968). A larger value of D_l was used above the transition region to be consistent with the observed increase in head-group thickness (see Figure 2; compare curves A and B). As noted in the text, their method assumes the mass density of the bilayer to be 1 which is not necessarily consistent with our results. Fortunately, our results are not strongly dependent on the precise value of D_l so that the Luzzati method is a reasonable way of avoiding an arbitrary choice. D_m, the width of the methyl trough, was based upon the width reported by White et al. (1981). This is a much stronger assumption because White et al. showed that the hexane is well localized to the central 10 Å of the bilayer largely independent of hexane concentration. Further, because the scattering lengths of the acyl chains and hexane are very close to one another, the precise dimensions of the trough have little effect on the results. For both assumptions, we have the additional fortunate circumstance that the calculational procedures tend to conserve the area under the curve. Thus, small errors in D_l or D_m are compensated for by appropriate changes in the ρ's. When the dust settles, we end up with a very accurate prediction of D_h which is little affected by the choices of D_l and D_m. This is fine for our purposes because D_h is really the only parameter we need to know accurately to arrive at the data in Table I.

The strip-model method of calculating structural parameters as used in this paper is a rather simple one; we have used as little data and as few assumptions as possible for a very limited objective. The method can be used much more powerfully to learn much more about bilayer structure. A complete presentation of the details of this approach will be presented in a separate publication where the question of the uniqueness of the models will also be addressed in detail. Our analysis shows that we can indeed determine structural parameters uniquely. The major problem in the present paper was to determine D_h. For $X_M = 0$, we have 8 orders of diffraction data (unpublished results), which is more than sufficient to fix uniquely the six parameters of the model. The D_h we calculate using the 8 orders of data is not significantly different from the value calculated by using 4 orders.

A question of major importance is what molecular components are included in a particular strip. The detailed analysis indicates that the strongly scattering carbonyl groups lie just outside the hydrocarbon core strip. We confirmed this prediction by measuring the transbilayer spacing of acyl chain carbons deuterated in the C-2 positions (see Discussion). Because the models are a means of averaging and smoothing, one must be careful not to overinterpret data with them. It is for this reason that we have not proposed detailed models for the interaction of the hexane with the bilayer. We can only say with certainty that the volume of the hydrocarbon core appears to remain constant as hexane enters it. We cannot say precisely how, for example, the head groups and water rearrange themselves as the hexane is accommodated by the system because the strip models are practically useless in this region. The "fluid layer" ($D - D_l$) is quite thin and beyond our resolution of about 6 Å ($=D/2h$ where $h = 4$). We cannot, therefore, rely on the strip models to learn much about the polar region. This is not true for the hydrocarbon region, on the other hand, because its width is much greater than our resolution limit.

Registry No. DOPC, 10015-85-7; $CH_3(CH_2)_4CH_3$, 110-54-3; neutron, 12586-31-1.

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Interaction of Duramycin with Artificial and Natural Membranes†

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Received October 9, 1984

ABSTRACT: Duramycin is a polypeptide antibiotic (molecular weight 2012) obtained from culture filtrates of *Streptomyces cinnamomeus* forma azacoluta. In this work, we show that low concentrations of duramycin induced aggregation of lipid vesicles containing unsaturated phosphatidylethanolamine and unsaturated monogalactosyl diglyceride, and of sarcoplasmic reticulum vesicles from rabbit skeletal muscle. Furthermore, duramycin inhibited the ATP-dependent Ca^{2+} uptake in sarcoplasmic reticulum vesicles without affecting the hydrolysis of ATP or the permeability of Ca^{2+}. Also, duramycin only inhibited the bacteriorhodopsin proton pump reconstituted into phospholipid vesicles containing phosphatidylethanolamine. We have isolated a duramycin-resistant strain of *Bacillus subtilis* and have mapped the location of duramycin resistance. In this strain, the secretion of protons and influx of calcium were resistant to duramycin, and its lipid composition was profoundly different from that of the parent strain. No phosphatidylethanolamine was detected in the resistant strain. Our findings are consistent with the idea that duramycin recognizes a particular membrane conformation determined by the presence of phosphatidylethanolamine or monogalactosyl diglyceride.

Duramycin is a polypeptide antibiotic (M_r 2012) obtained from culture filtrates of *Streptomyces cinnamomeus* forma azacoluta (NRRL B-1699). It is active against Gram-positive bacteria, some yeasts, and fungi (Shotwell et al., 1958). It was shown recently that duramycin inhibits at low concentrations (5 μg/100 μg of protein) the chloride transporter and at higher concentrations (20 μg/100 μg of protein) the proton pump of clathrin-coated vesicles (Stone et al., 1984). It also permeabilizes intact cells (Racker et al., 1984). At very high concentrations (150 μg/100 μg of protein), the (Na^+, K^+)-ATPase was significantly inhibited, and reversal was observed by lipid mixtures containing phosphatidylethanolamine (Nakamura & Racker, 1984).

In this paper, we describe experiments on the interaction of duramycin with artificial liposomes, with the Ca^{2+} pump of sarcoplasmic reticulum, and with the proton pump of vesicles reconstituted with bacteriorhodopsin and the secretion of protons and uptake of calcium in duramycin-sensitive and duramycin-resistant *Bacillus subtilis*. Our data suggest that

† This investigation was supported by U.S. Public Health Service Grant CA-08964, awarded by the National Cancer Institute, DHHS, and the American Heart Association, New York Chapters (Southern Tier, Northern, and Dutchess County).

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0006-2960/85/0424-4645$01.50/0