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Solute Partitioning into Lipid Bilayer Membranes†

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ABSTRACT: We have measured the membrane/water partition coefficients of benzene into lipid bilayers as a function of the surface density of the phospholipid chains. A simple ^2H NMR method was used for the measurement of surface densities; it is shown to give results similar to those obtained from more demanding X-ray diffraction measurements. We observe that benzene partitioning into the bilayer is dependent not only on the partitioning chemistry, characterized by the oil/water partition coefficient, but also on the surface density of the bilayer chains. Increasing surface density leads to solute exclusion: benzene partitioning decreases by an order of magnitude as the surface density increases from 50% to 90% of its maximum value, a range readily accessible in bilayers and biomembranes under physiological conditions. This effect is independent of the nature of the agent used to alter surface density: temperature, cholesterol, and phospholipid chain length were tested here. These observations support the recent statistical thermodynamic theory of solute partitioning into chain molecule interphases, which predicts that the expulsion of solute is due to entropic effects of the orientational ordering among the phospholipid chains. We conclude that the partitioning of solutes into bilayer membranes, which are interfacial phases, is of a fundamentally different nature than partitioning into bulk oil and octanol phases.

The partitioning of solute molecules into lipid bilayers and biological membranes is the basis for drug and metabolite uptake and passive transport across membranes and may be involved in the molecular mechanism of anesthetic drug action. In related interfacial phases, where short chains are likewise confined at high density to an interface, solute partitioning processes underly micellar stability and catalysis and selectivity and retention in reverse-phase liquid chromatography (Dill, 1987).

This partitioning process has often been characterized with bulk thermodynamic models as though bilayer membranes were identical with bulk phases. Lipid bilayer membranes, however, have high surface to volume ratios, they are interfacial phases of matter. In interfacial phases physical properties vary with distance from the interface. In contrast, in bulk phases physical properties are uniform throughout. For example, there is a gradient of chain disorder in the hydrocarbon core of the bilayer: the surfactant chains are most highly aligned near the headgroups, and the order diminishes with distance toward midbilayer (Hubbell & McConnell, 1971; Seelig, 1977; Dill & Flory, 1980). Moreover, the chain ordering of the bilayer phospholipids increases with surface

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density. Properties of interfacial phases depend on surface density whereas properties of bulk phases do not.

These structural differences between bilayers and other interfacial phases and bulk phases such as oil or octanol should be manifested as differences in the nature of solute partitioning into them. Recent theory (Marqusee & Dill, 1986) has predicted the following: (i) There will be an equilibrium gradient of solute concentration in the bilayer in contrast to the uniform distribution expected in a bulk phase; this prediction is consistent with neutron scattering experiments (White et al., 1981) and experiments on planar bilayers (Andrews et al., 1970; Brooks et al., 1975; White, 1977). (ii) The partial chain ordering should disfavor solute retention in the bilayer relative to amorphous bulk phases. (iii) The solute uptake should decrease significantly with increased surface density of the chains. There is some suggestive, but indirect, evidence in support of the latter two conclusions. The partition coefficients of many anesthetics into membranes are 2–15-fold lower than their coefficients of partitioning into olive oil (Seeman, 1972). Entropies of transfer of short-chain hydrocarbons (Miller et al., 1977; Simon et al., 1977) and noble gases (Katz & Diamond, 1974) into bilayers are more negative than that of transfer into amorphous hydrocarbon. Also, factors which cause increased surface density of the bilayer, such as decreasing the temperature through T_c,[1] the main phase transition temperature of the phospholipid (Antunes-Madeira & Madeira, 1985; Luxnat & Galla, 1986; Simon et al., 1979), or incorporation of cholesterol above T_c (Antunes-Madeira, 1985; Luxnat & Galla, 1986; Miller & Yu, 1977; Miller et al., 1977; Simon & Gutknecht, 1980; Simon et al., 1977; Smith et al., 1981), lead to lower partition coefficients. The pioneering work of Simon and co-workers laid the groundwork for the present studies.

Here we have undertaken a systematic experimental investigation of the coefficient of partitioning of benzene from an aqueous medium into various bilayer membranes as a function of their surface densities. A simple ^{2}H NMR method is used for the measurement of bilayer surface density. A similar approach has previously been reported by Seelig and others (Schindler & Seelig, 1975; Seelig & Seelig, 1974). We control the surface density using temperature, phospholipid chain length, and the incorporation of cholesterol. The experiments show that benzene partitioning decreases by an order of magnitude with increasing surface density over a range of surface densities readily accessible to biological membranes. We also show that this solute expulsion is increased by increased surface density of the chains and is independent of the nature of the agent used to control the surface density.

Materials and Methods

Materials. Dilauroylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), and dipalmitoylphosphatidylcholine (DPPC), which were protiated or perdeuteriated along the acyl chains, and protiated egg phosphatidylcholine (egg PC) were purchased from Avanti Polar Lipids and used without further purification. Cholesterol was obtained from Sigma, and ultrapure octane, hexadecane, and benzene were obtained from Aldrich. [U-^{14}C]Benzene was purchased from Amersham Corp. (sp act. 50–125 mCi/mmol) and diluted with benzene to a specific activity of approximately 10^5 cpm/μL.

Vesicle Preparation. Protiated multilamellar vesicles (MLVs) were prepared by coevaporating phospholipid and cholesterol (0–40 mol %) followed by a 2-h vacuum evacuation. The lipid was rehydrated with 0.1 M NaCl and 10^{-4} M EDTA to a concentration of 50 μmol/mL total lipid. The lipid was then vortexed 5 min above T_c, incubated above T_c for 16 h, and stored at 5 °C under nitrogen. No dependence on incubation time (1–24 h), cycling through T_c, or alternate MPV preparation (Gruner, 1985) was seen in the partition coefficient, K. Phospholipid concentration was determined by a modified Bartlett phosphate assay (Bartlett, 1959).

Perdeuteriated DLPC, DMPC, and DPPC MLVs were prepared in the same manner as the protiated MLVs except the lipid was resuspended in 0.1 M NaCl–10^{-4} M EDTA in ^{2}H-depleted water. The MLVs were centrifuged for 10 min at 15 000 rpm and resuspended to remove small vesicles which would give an isotropic NMR signal.

Measurement of Surface Density. The area per phospholipid molecule was determined by deuterium NMR. We placed 0.4 mL of 10–20 μmol/mL perdeuteriated phospholipid with 0–40 mol % cholesterol in the cylindrical solid sample cell, 8 mm diameter × 23 mm. Spectra were taken at 46.07 MHz in a GE GN300 spectrometer interfaced with a Nicolet Model 2090 fast digitizer. A Fourier transform quadrupolar echo technique (Davis et al., 1976) with 3.5-μs 90° pulses separated by 50-μs delays was used to obtain a powder pattern in 1000–2400 acquisitions. The spectral width was 500 kHz, corresponding to a dwell time of 1 μs. A total of 4K data points were collected, and the FID was left shifted to the top of the echo. There were no phase-shift errors between the transmitter and receiver signals. Spectra were collected between 10 and 55 (±1) °C. Thermal equilibration was achieved within 10 min of temperature changes. The average surface density from two or more determinations is reported; errors were within 3% of the mean value. The normalized surface density σ is reported, being defined as the ratio of the area per phospholipid molecule in the crystal, A_0 = 40.8 Å^2, to the area per phospholipid, A.

^{2}H NMR is commonly used to determine the order parameter profile of the acyl chain methylene groups in the bilayer. The plateau value of this profile can be used to calculate surface density, as shown below. The premise is that there is a direct relationship between the surface density and the chain organization, characterized by the order parameter profile. This premise is supported by experiments of Mely et al. (1975). They have shown that two potassium laurate bilayer preparations of different hydration and at different temperatures, but with the same area/molecule, have the same order parameter profiles. The disorder gradient can also be predicted from surface density and chain length (Dill & Flory, 1980). The surface density of the bilayer phospholipids can be determined from the maximum quadrupolar splitting in the ^{2}H NMR spectrum, which corresponds to the plateau value of the order parameter (Davis, 1979). We do so as follows. In a powder pattern the separation between peak maxima, the quadrupolar splitting ($\Delta\nu_Q$), is related to the molecular order parameter S_{mol} by (Seelig, 1977)

$$\Delta\nu_Q = \frac{3}{8}\left(\frac{e^2qQ}{h}\right)S_{mol} \qquad (1)$$

S_{mol} is related to the C–D bond order parameter S_{CD} by $S_{mol} = -2S_{CD}$. S_{mol} is defined in terms of β, the angle between the midpoint of the C–C bond of interest in the acyl chain and

[1] Abbreviations: T_c, phospholipid main phase transition temperature; egg PC, egg yolk phosphatidylcholine; DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; X_{chol}, mole fraction cholesterol; K, mole fraction partition coefficient; MLVs, multilamellar vesicles.

the bilayer normal (Seelig, 1977):

$$S_{mol} = \frac{3\langle\cos^2\beta\rangle - 1}{2} \quad (2)$$

The relationship between $\langle\cos^2\beta\rangle$ and σ is established through use of the lattice theory of chain packing (Dill & Flory, 1980; Dill, 1984). Accordingly, chain bonds are classified as either approximately parallel ($\parallel$) with the bilayer normal ($\beta = 0°$) or perpendicular ($\perp$) to it ($\beta = 90°$). Therefore, the ensemble average at depth z from the interface is simply

$$\langle\cos^2\beta\rangle_z = \frac{(\text{no. of } \parallel \text{ bonds})_z \cos^2 0° + (\text{no. of } \perp \text{ bonds})_z \cos^2 90°}{(\text{no. of } \parallel \text{ bonds})_z + (\text{no. of } \perp \text{ bonds})_z} \quad (3)$$

$$= p_z = \frac{(\parallel)_z}{(\parallel)_z + (\perp)_z} \quad (4)$$

the fraction of all bonds at depth z which are parallel, since $\cos^2 90° = 0$ and $\cos^2 0° = 1$.

According to the theory (Dill & Flory, 1980; Dill, 1984), $p_1 = p_{plateau}$ is calculated as the solution to

$$(1 - p_1)^n + (1 - p_1)^{n-1} + (1 - p_1)^{n-2} + \ldots + (1 - p_1) + 1 = 1/\sigma \quad (5)$$

where n is the number of segments per chain. For chains of sufficient length that a plateau in the order exists, we can use the approximation $1 + x + x^2 + x^3 + \ldots + x^n \approx 1/(1 - x)$, and eq 5 simply becomes

$$1/p_1 = 1/\sigma \quad (6)$$

Combining eq 4 and 6 leads to

$$\langle\cos^2\beta\rangle_{plateau} = \sigma = \frac{A_0}{A} \quad (7)$$

Taking the value of $e^2qQ/h = 170$ kHz (Burnett & Muller, 1971), the normalized surface density is thus given in terms of the plateau region quadrupolar splitting as

$$\sigma = \frac{2}{3}S_{mol} + \frac{1}{3} = \frac{16}{9}\left(\frac{\Delta\nu_{Q(plateau)}}{170\text{ kHz}}\right) + \frac{1}{3} \quad (8)$$

where $\Delta\nu_{Q(plateau)}$ is the maximum quadrupolar splitting in the spectrum. The molecular areas determined in this manner are comparable to those determined by X-ray diffraction measurements as described below. Hence, we adopt the combination of ^{2}H NMR and eq 8 above for the purpose of determining surface densities of the bilayer phospholipids.

Calculations of phospholipid areas from ^{2}H NMR quadrupolar splittings have previously been reported (Schindler & Seelig, 1975; Seelig & Seelig, 1974). However, those comparisons are derived from the use of an empirical model (Marčelja, 1974) inappropriately based on the assumption that anisotropic attractions are responsible for chain organization [for a discussion of this assumption, see Dill (1984), Gelbart and Gelbart (1977), and Warner (1980)] and based on the use of an adjustable parameter to fit the order parameters. The approach outlined here does not require free parameters and is based on the more appropriate physical model that anisotropy arises from steric repulsions.

Partition Coefficient. A gas-phase equilibration of radiolabeled benzene, in the simple bottle system shown in Figure 1, was used to determine benzene partition coefficients. This method is similar to others previously published (Wishnia & Pinder, 1966; Simon et al., 1982). We placed 3 mL of H_2O and two glass vials containing a small magnetic stir bar and 250 μL of either 0.1 M NaCl–10^{-4} M EDTA or MLVs in the same aqueous solution into a 70-mL glass bottle with a

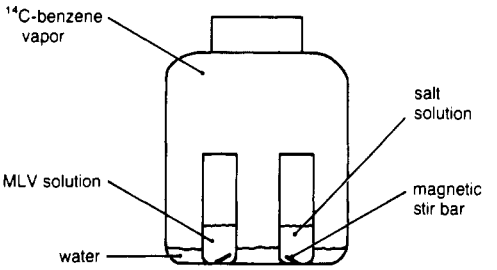

FIGURE 1: Experimental apparatus used to measure partition coefficients. The vesicle solution and corresponding aqueous solution are each placed in a glass vial along with a magnetic stir bar. Water surrounds the vials to facilitate equilibration with the external water bath. Radiolabeled benzene is pipeted into the bottle and equilibrates through the vapor phase. A TFE-lined cap prevents vapor leakage. The bottles are placed on a magnetic stirrer, submerged in a water bath, and incubated at fixed temperature.

TFE-lined cap to prevent gas leakage. There was no dependence of K on the volume of solution inside the vials, up to 500 μL. In experiments above 35 °C, the bottle and water were preequilibrated to saturate the gas phase with water. For oil/water partitioning, 250 μL of the water-saturated oil or oil-saturated water was placed in the vials. A small volume (0.25–36 μL) of the radiolabeled benzene was pipetted onto the side of the glass bottle which was then quickly capped. To avoid oxidation of its unsaturated acyl chains, egg PC studies were done under nitrogen. The bottles were stirred on a 15-position magnetic stirrer submerged in a water bath. Equilibrium was reached within 2 or 4 h for samples above and below the T_c of the pure phospholipid, respectively, regardless of the cholesterol concentration. For the lowest temperatures in DMPC and DPPC, stirring was intermittent to prevent foaming of the lipid. For oil/water partition coefficients samples were equilibrated for 6 h.

An aliquot was sampled from each vial and transferred directly into scintillation fluid. The aliquots cannot be placed into the scintillation vial first with later addition of scintillation fluid as this causes benzene to adsorb irreversibly to the glass surface of the scintillation vial. Mole fraction partition coefficients were calculated after correction for the cpm in the aqueous fraction of the MLV solution; lipid densities were taken from Nagle and Wilkinson (1978). Mole fraction partition coefficients are only appropriate for solute molecules whose size is the same as that of the solvents. However, it is currently unclear what lipid volume is appropriate to use in the volume fraction partition coefficient. The benzene distribution within the membrane likely changes with surface density (Marqusee & Dill, 1986). Moreover, surface adsorption can also contribute to partitioning. Therefore, some headgroup region, in addition to the hydrocarbon core, may be the relevant partitioning volume. Statistical thermodynamic theory is presently being developed to define the appropriate concentration variable for bilayer systems with solute and solvent molecules of different size (Naghizadeh and Dill, unpublished results). Little benzene is lost during sampling as determined by (i) investigation of the time dependence of sampling and (ii) the correspondence with literature values (see below) of partition coefficients and the Henry's law constant for benzene in water.

The dependence of K on benzene concentration was determined for DMPC at 5, 15, and 30 °C, DMPC with 15 and 40 mol % cholesterol at 10 and 40 °C, and egg PC and DPPC at 25 °C. For comparison with surface densities obtained in the corresponding perdeuteriated MLVs, the temperature dependence of partitioning was studied in DLPC, DMPC, and DPPC MLVs with 0–40 mol % cholesterol in the temperature

Table I: Comparison of Phospholipid Molecular Areas Determined by ^2H NMR and X-ray Diffraction

		area/phospholipid (Å^2)					
		^2H NMR		X-ray diffraction[a]			
phospholipid	temp (°C)	this study[b]	Schindler & Seelig (1975)[c]	Janiak et al. (1976, 1979)	Lis et al. (1982)	Lewis & Engelman (1983)[d]	Hui & He (1983)
DLPC	25	71			69	66	
DMPC	10–15			50			
	25	67		59	65		
	35	70		62		66	61,[e] 66[f]
	45	72		62			
DPPC	25			49	52		
	41		59				
	45	68 (64)		67		66	
	50	69 (67)	61	68	71		
	55–57	71 (69)	63	69			
	64			70			

[a] Phospholipid areas for MLVs in excess water (except Lewis and Engelman), calculated from the equations of Rand et al. (1975). [b] Values in parentheses calculated from eq 8 and the average S_{mol} of C2–C9 from Seelig and Seelig (1974). [c] Areas calculated from the equations of Schindler and Seelig (1975) and the ^2H NMR data of Seelig and Seelig (1974). [d] Data for small unilamellar vesicles in excess water. Specific hydration data not needed with their method. [e] Calculated from the hydration data of Janiak et al. (1976). [f] Calculated from the hydration data of Lis et al. (1982).

range 10–55 °C. The mole fraction of benzene in the lipid was ≤0.025 in these studies. This gives K values within experimental error of their projected infinite-dilution values. Benzene partition coefficients at this "infinite dilution" value are compared with surface densities determined in the perdeuteriated phospholipid at the same temperature and cholesterol concentration with no benzene present.

Results and Discussion

Surface Density Determination. Representative powder patterns for perdeuteriated DMPC, 30 mol % cholesterol, at 11 and 50 °C are shown in Figure 2. It is evident from the figure that $\Delta\nu_Q$ decreases with increasing temperature, indicating a decrease in the ordering or increase in the area per phospholipid molecule. Below the main phase transition temperature of the pure phospholipid, a broadened ^2H NMR spectrum is obtained which is not interpretable by our analysis. The addition of cholesterol below the T_c disorders the membrane to an extent that results in a powder pattern. Therefore, surface densities can be determined below T_c only if cholesterol is present.

Our values of order parameters are in agreement with those previously measured. The reported plateau value of S_{mol} = 0.40 for perdeuteriated DPPC at 45 °C (Davis, 1979) agrees well with the value of 0.395 obtained in this study. Our observation that the main phase transition temperature of the perdeuteriated phospholipid is 5° lower than that of the protiated lipid also agrees with earlier studies (Peterson et al., 1975). The quadrupolar splittings obtained in this study correspond closely to those obtained by Jacobs and Oldfield (1979) for [3′,3′-D_2]DMPC labeled on the *sn*-2 chain at similar temperatures and cholesterol concentrations.

Shown in Figure 3 is the dependence of the normalized surface density on temperature and cholesterol concentration for DLPC, DMPC, and DPPC. The surface density is observed to increase with increasing cholesterol concentration and to decrease with increasing temperature over a range of σ = 0.53–0.91 at temperatures above T_c. High surface densities with little dependence on cholesterol concentration are seen below T_c.

Comparison with X-ray Diffraction. To confirm the validity of the ^2H NMR technique for determining the average phospholipid area, A, a comparison was made with X-ray diffraction data in the literature; these data are shown in Tables I and II. X-ray d spacings are consistent among investigators. However, when reported, the lipid weight fractions at full hydration, necessary to calculate A, are highly variable. The equations of Rand et al. (1980) are used to calculate area per phospholipid molecule from the measured d spacings and lipid hydration. The data of Lewis and Engelman (1983) are for sonicated vesicles. The technique they used did not require that the weight fraction of the lipid be known.

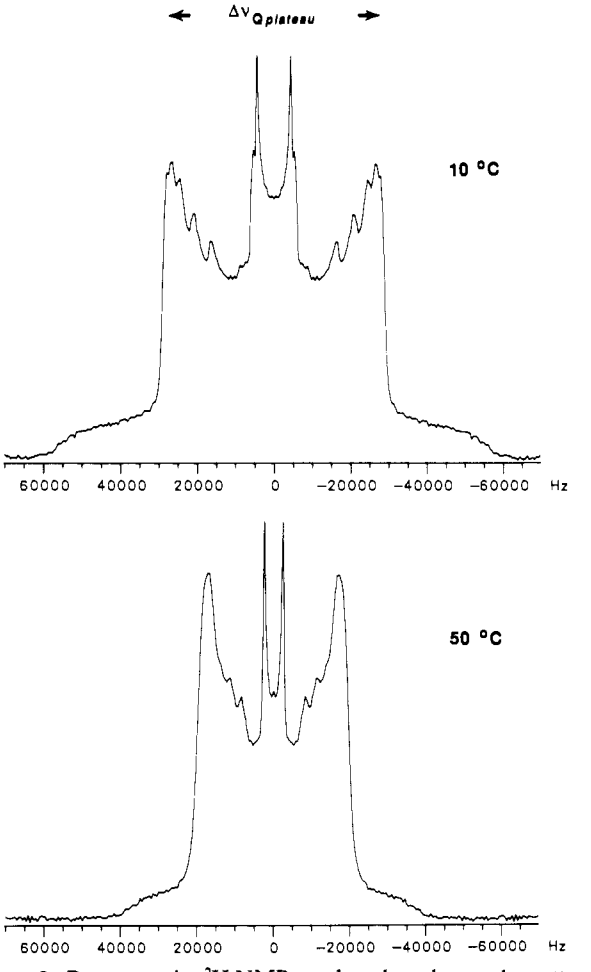

FIGURE 2: Representative ^2H NMR quadrupolar echo powder patterns of perdeuteriated DMPC with 30 mol % cholesterol at 10 and 50 °C. $\Delta\nu_{Q(plateau)}$, the quadrupolar splitting between the outermost peaks, decreases with increasing temperature corresponding to decreased order of the acyl chains. Symmetrical spectra were obtained by reflecting about the central frequency.

Table II: Comparison of Phospholipid Areas Determined by ^2H NMR and X-ray Diffraction for Cholesterol-Containing Membranes below T_c

phospho-lipid	temp (°C)	X_{chol}	area/phospholipid (Å^2)[a] ^{2}H NMR (this study)	X-ray diffraction[b] Lis et al. (1982)	Ladbrooke & Chapman (1968)	Hui & He (1983)
DMPC	10	0.1				(52)
		0.2	46			(55)
DPPC	25	0.1		49	41 (51)	
		0.2	46			
		0.4	46		42 (57)	
		0.5		60	43 (60)	

[a] Area per phospholipid only. Area from associated cholesterol molecules has been subtracted (see text). Phospholipid areas for MLVs in excess water calculated from the equations of Rand et al. (1975). [b] Area values in parentheses calculated with a hydration value of 0.42 (see text).

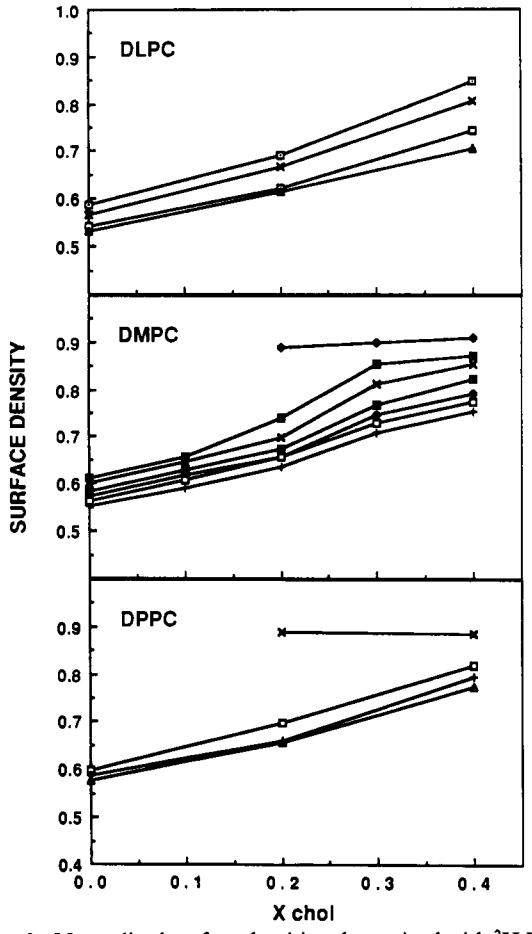

FIGURE 3: Normalized surface densities, determined with ^2H NMR, as a function of cholesterol mole fraction at various temperatures: DLPC at (⊡) 20, (×) 29, (□) 45, and (▲) 55 °C; DMPC at (◆) 11, (■) 25, (×) 30, (■) 35, (◇) 40, (□) 45, and (+) 50 °C; and DPPC at (×) 30, (□) 45, (+) 50, and (▲) 55 °C. The main phase transition temperatures, T_c, of the pure phospholipids are 0, 23.5, and 41.5 °C for DLPC, DMPC, and DPPC, respectively. Above T_c, surface density increases with increasing cholesterol concentration and decreasing temperature. Below T_c, surface density changes little with cholesterol concentration.

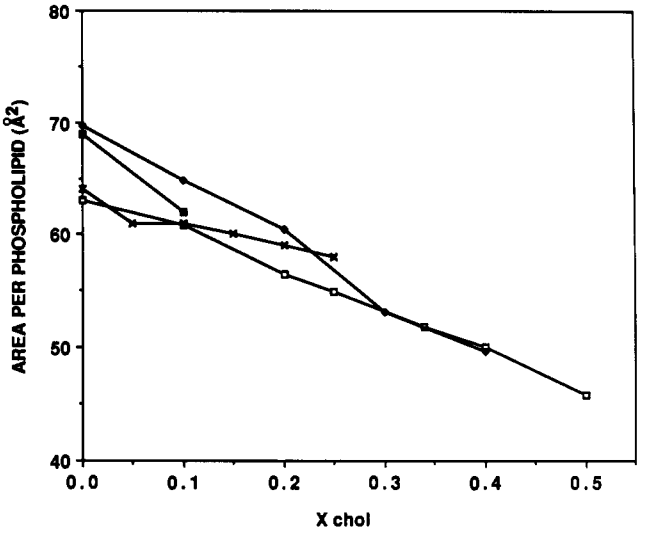

FIGURE 4: Areas per phospholipid molecule vs cholesterol concentration determined by X-ray diffraction and ^2H NMR: ^2H NMR areas for DMPC at (◆) 35 °C; X-ray diffraction areas for (□) egg PC at ambient temperature and 35% H_2O (Lecuyer & Dervichian, 1969), (×) DMPC at full hydration at 35 °C (Hui & He, 1983), and (■) DPPC at full hydration at 45 °C [0% cholesterol (Janiak et al., 1976); 10% cholesterol (Rand et al., 1975)]. The trend in area per molecule is similar for all lipids and temperatures shown.

As shown in Table I, the areas determined by X-ray diffraction and ^2H NMR, for pure DPPC above T_c, are in excellent agreement. With DLPC and DMPC the NMR areas are consistently larger than the X-ray values. However, they appear to be within the experimental error over the range of X-ray diffraction values reported. The DMPC and DPPC thermal area expansion coefficients, $c_T = \Delta A/(A\Delta T)$, above T_c are 3.6×10^{-3} and 4.3×10^{-3} °C^{-1}, respectively, in this study and 2.8×10^{-3} and 3.1×10^{-3} °C^{-1}, respectively, for the X-ray data of Janiak et al. (1976). Using a micromechanical technique, Evans and Kwok (1982) obtained a comparable value of 4×10^{-3} °C^{-1} for DMPC above T_c.

Also shown in Table I are the DPPC areas calculated from the ^2H NMR quadrupolar splittings of Seelig and Seelig (1974) using the equations of Schindler and Seelig (1975). For this purpose S_{mol} is taken to be equal to $-2S_{CD}$. Their method correctly calculates the change in phospholipid area with temperature, $c_T = 3.2 \times 10^{-3}$ °C^{-1} but does not accurately predict the phospholipid areas. The S_{mol} values in the plateau region of the Seelig and Seelig (1974) study are somewhat larger than those obtained in this work. Included in parentheses in Table I are area values determined with eq 8 and the mean S_{mol} value of the plateau region C2–C9 carbons from the Seelig study. The more rigorous calculation presented here appears to be better able to determine phospholipid areas from ^2H NMR data.

Figure 4 shows the dependence of phospholipid area on cholesterol concentration from X-ray diffraction measurements for DMPC at 35 °C (Hui & He, 1983), DPPC at 45 °C (Janiak et al., 1976; Rand et al., 1975), and egg PC at ambient temperature (Lecuyer & Dervichian, 1969) and our NMR areas for DMPC at 35 °C. These are areas per phospholipid molecule; the area from associated cholesterol molecules has been subtracted in the manner of Rand et al. (1975) with 37 Å^2 as the area per cholesterol molecule (Lecuyer & Dervichian, 1969). For the DMPC and DPPC bilayers containing cholesterol, the value of the weight fraction of lipid, c, at full

hydration was taken to be 0.58 for all cholesterol concentrations. Lis et al. (1982) found c to be 0.58 for DPPC/cholesterol at 1:1 and 8:1 mole ratios and 0.60 for egg PC/cholesterol at 1:1, all at 25 °C. Hydration data for pure and cholesterol-containing phospholipids has also been collected by Elworthy (1962) and Jendrasiak and Mendible (1976). Their c values tend to match each others but are larger than those of Janiak et al. (1976) and Lis et al. (1982). The egg PC/cholesterol bilayers at ambient temperature contain 35% water, less than full hydration (Lecuyer & Dervichian, 1969). Above the T_c of the pure phospholipid, the correlation of the NMR areas with those of X-ray diffraction is reasonably good for cholesterol-containing membranes. For DMPC the NMR areas are slightly higher than those of Hui and He (1983). However, the overall trend for all the phospholipids and both techniques is similar.

Below T_c only areas from cholesterol-containing membranes can be calculated from ^{2}H NMR. The correlation of the NMR and X-ray diffraction data at these temperatures is shown in Table II. The NMR data lie within the range of the X-ray literature values, but little change is seen in the NMR areas on increasing the cholesterol concentration from 20 to 40 mol %, in contrast to the X-ray diffraction areas. However, the variation in hydration values between investigators again makes interpretation difficult. Some difference may result from the inability of the NMR technique to account for chain tilt below T_c. Note, however, that this discrepancy does not obstruct our ultimate purpose of correlating benzene partitioning with surface density, inasmuch as only the highest surface densities, above 0.89, are from bilayers below T_c. Areas for egg PC/cholesterol membranes were not calculated from the ^{2}H NMR data of Stockton and Smith (1976) for comparison with the X-ray diffraction data of Lecuyer and Dervichian (1969). There is evidence that the deuteriated fatty acid probes used in the NMR study significantly alter bilayer properties (Pauls et al., 1983).

Subject to the caveats above, the correlation of phospholipid areas determined by X-ray diffraction and ^{2}H NMR is quite good. Also, the trends in the areas derived from ^{2}H NMR as a function of temperature and cholesterol are consistent with those of X-ray diffraction. We use the areas so determined in the following comparison of benzene bilayer partitioning with surface density.

Partitioning Experiments. We have measured the bulk phase partitioning of benzene between water and several amorphous hydrocarbons for comparison with other experiments reported in the literature. The partitioning of benzene from water to benzene at 25 °C was determined to be 2397 ± 279 (SD) which is in good agreement with values of 2493 and 2425 reported respectively by McAuliffe (1986) and Gill et al. (1976). The partition coefficients for benzene between octane and water and between hexadecane and water at 25 °C are 1494 ± 14 and 1993 ± 34, respectively. The larger partitioning into hexadecane is expected, on the basis of Flory–Huggins theory, due to the larger volume of the hexadecane molecule (Fung & Higuchi, 1971; De Young and Dill, unpublished results). Reported values for benzene n-alkane/water partition coefficients at 20–25 °C are as follows: hexane/water, 1006 (Ben-Naim & Wilf, 1979) and 2057 (Sekine et al., 1973); heptane/water, 1594 (Suzuki et al., 1982); hexadecane/water, 1500 (Simon et al., 1982). The volume fraction partition coefficients of these investigators were converted to mole fraction units by assuming a low benzene concentration. Our data fall within this wide range of values. All our data were collected in the Henry's law region. If the benzene pressure in the bottle partitioning system is calculated with the ideal gas law, assuming no benzene is lost, then the Henry's law constant is K_H = 330 ± 30 atm at 25 °C. This agrees with literature values of 305–360 atm (Hine & Mookerjee, 1975; Pierotti, 1965; Simon et al., 1982).

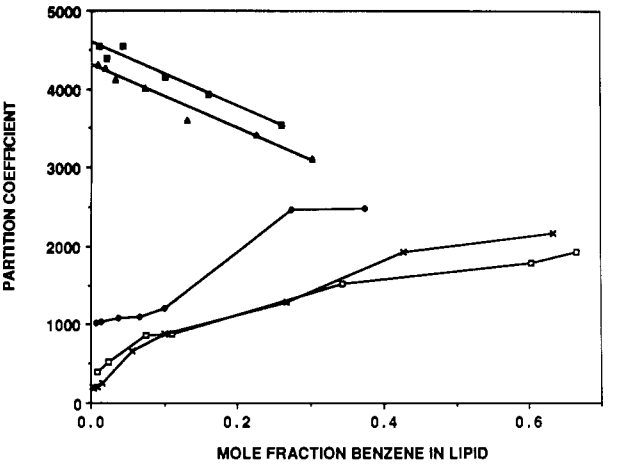

FIGURE 5: Dependence of membrane/water partition coefficients on the mole fraction of benzene in the lipid phase for various lipids and temperatures: DMPC at (×) 5, (◆) 15, and (▲) 30 °C; DPPC at (□) 25 °C; egg PC at (■) 25 °C. For lipids above their T_c's, K decreases linearly with increasing benzene concentration. Below T_c, K shows a strong positive dependence on benzene concentration. Large deviations of K from its infinite-dilution value are seen at higher benzene concentrations.

Concentration Dependence of Partitioning. The concentration dependence of benzene partitioning into phosphatidylcholine vesicles at various temperatures is shown in Figure 5. Mole fraction partition coefficients are reported as a function of the mole fraction of benzene associated with the lipid phase. It is evident that temperature and phospholipid type have a significant effect on the concentration dependence of partitioning. Egg PC at 25 °C ($T_c \approx -10$ °C) and DMPC at 30 °C (T_c = 23 °C), both above their T_c's, show a decrease in K with increasing benzene concentration. In contrast, in DMPC at 15 °C the partition coefficient increases with benzene concentration. The addition of a large enough quantity of benzene causes melting of the frozen bilayer. This has also been observed previously with Raman spectroscopy (Szalontai, 1976). In DMPC at 5 °C and DPPC at 25 °C (T_c = 41 °C) a very rapid rise in K is seen at very low benzene concentrations. This may be the result of benzene interacting in the headgroup region at the hydrocarbon/water interface of the vesicle, rather than a result of partitioning into the hydrocarbon core. The existence of this strong concentration dependence and the inability to obtain the surface density of these pure phospholipid systems below their T_c's directly from the ^{2}H NMR spectra suggest that these systems cannot be analyzed by the methods we have used here. We do not include these data in subsequent figures. DMPC MLVs containing 15 and 40 mol % cholesterol showed no concentration dependence of partitioning at 10 or 40 °C, temperatures below and above the T_c of the pure phospholipid (data not shown). We believe an error in the report of Simon et al. (1982) leads to their partition coefficients not appearing to agree with our infinite-dilution values. They reported that the partial pressure of benzene was about 30 mmHg for most of their experiments and that the mole fraction of benzene in the lipid was quite small, approximately 0.1. These statements are inconsistent with each other. Using their reported Henry's law constant for benzene of 360 atm at 25 °C, the mole fraction of benzene in water would be 1.1×10^{-4}. They report

the partition coefficient of benzene into egg PC at 25 °C to be 3120. The mole fraction of benzene in the lipid in their experiments is therefore approximately 0.34, rather than 0.1. At this benzene concentration in the lipid, our partition coefficient into egg PC is 3200, which agrees with their value at that same concentration. However, we find the partition coefficient at infinite dilution to be 4600, which implies that their infinite-dilution concentration is somewhat too high and leads to underestimation of the true infinite-dilution partition coefficient. DLPC and DMPC partition coefficients also agree with those reported by Simon et al. at similar benzene concentrations in the lipid phase. This does not, however, explain the difference between our strong dependence and their lack of a dependence of K on benzene concentration for DPPC at 25 °C.

For reasons evident in Figure 5, it is very important that partition coefficients be measured at, or extrapolated to, infinite dilution. We have found that for mole fractions ≤ 0.025 of benzene in the lipid the value of K is within the limits of experimental error of the infinite-dilution value. Therefore, in all subsequent experiments mole fractions of benzene in the lipid phase are ≤ 0.025. There is no concentration dependence in the bulk phase partitioning of benzene between hexadecane and water.

Cholesterol and Temperature Dependence of Partitioning. Benzene partitioning into DMPC membranes increases by approximately a factor of 8 when the temperature is increased from 5 to 30 °C, through T_c, as shown in Figure 5. This large change in partitioning on passing through the main phase transition temperature has been seen for several solutes, including hexane (Simon et al., 1979), lindane (Antunes-Madeira & Madeira, 1985), and chlorpromazine (Luxnat & Galla, 1986). This increase in partitioning is too large to be accounted for entirely by changes in oil or water solubilities; it must be largely due to the change in chain organization of the phospholipids.

The seemingly puzzling observation that benzene uptake into the bilayer at low surface densities is higher than that into oil (comparing the membrane/water partition coefficient to twice the oil/water partition coefficient, due to the pair of acyl chains per phospholipid) is readily explained by the propensity for benzene to adsorb at the headgroup interface (Ward et al., 1986). It has been shown that interfacial interactions [characterized by g in the Marqusee and Dill theory (1986)] can lead to very large concentration enhancements if the solute prefers the interface to either the aqueous or hydrocarbon environment. This difference in partitioning may also be due to molecular size and shape differences.

The partitioning of benzene into DLPC, DMPC, and DPPC MLVs as a function of cholesterol concentration is shown in Figure 6 for various temperatures. In each lipid, increased cholesterol concentration results in a decreased benzene partition coefficient at all temperatures above T_c. Below T_c little change in K is seen with cholesterol addition. Figure 6 also shows that partition coefficients are decreased by as much as a factor of 5 on addition of 40 mol % cholesterol. Our data were too limited to interpret the effect of cholesterol concentration on the ΔH and ΔS of transfer. DMPC and DPPC show increasing and then decreasing ΔH and ΔS values with increasing cholesterol concentrations whereas in DLPC ΔH and ΔS change little with cholesterol (data not shown).

Partitioning vs Surface Density. The principal purpose of these experiments is to determine the dependence of the infinite-dilution partition coefficient on the surface density of the bilayer chains. Temperature, cholesterol, and phospholipid

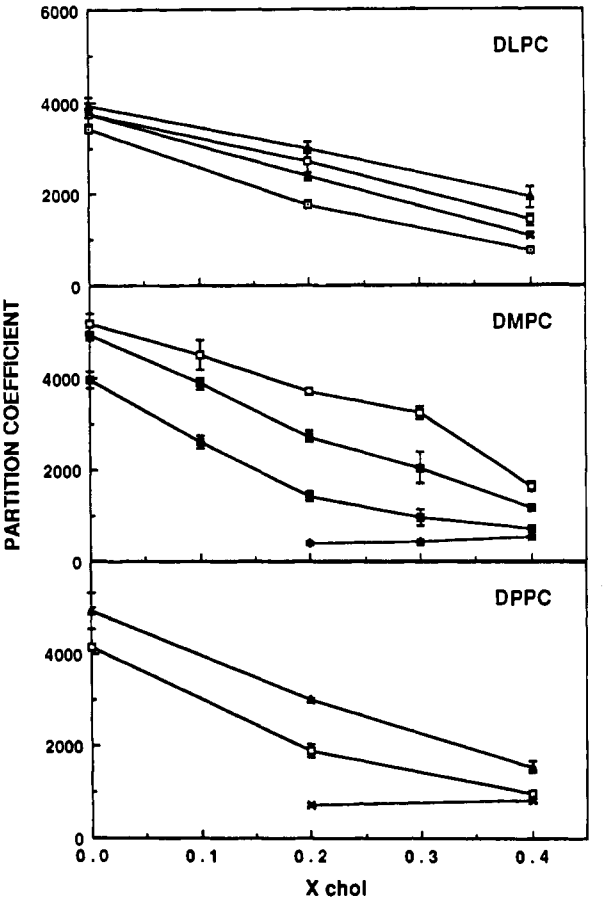

FIGURE 6: Dependence of the membrane/water partition coefficient of benzene on mole fraction of cholesterol in the membrane at various temperatures: DLPC at 20, 30, 45, and 55 °C; DMPC at 10, 25, 35, and 45 °C; DPPC at 30, 45, and 55 °C. Standard deviations are shown. Symbols are the same as those used in Figure 3. Benzene partitioning decreases with increasing cholesterol concentration at all temperatures above T_c for each of the phospholipids. Below T_c, a small partition coefficient with little dependence on cholesterol concentration is seen.

chain length were varied to obtain normalized surface densities between 0.53 and 0.91. In Figure 7 the dependence of K on surface density is shown for DLPC, DMPC, and DPPC. Each data point is the average of three or more samples; the error bars are standard deviations. In all three lipids, K decreases with increasing surface density. Increased chain ordering results in solute exclusion. It is clear that the effect of membrane surface density on partitioning is large; K decreases approximately 10-fold with a change in surface density from 0.5 to 0.9. Irrespective of whether the ordering agent was temperature or cholesterol, the same dependence of K on surface density is observed. In that regard, we have no evidence of specific association of benzene with the cholesterol molecules. This point is made more clearly in Figure 8, a composite of the three curves in Figure 7. The DMPC and DPPC data are superimposable over the entire range of surface density; the DLPC data are superimposable at surface densities above 0.65. DLPC differs from DMPC and DPPC at low surface densities; this may be due to a different DLPC physical structure. DLPC has a hydrocarbon chain length of 12, near the minimum length necessary to form bilayers. Near T_c the behavior of DLPC bilayers differs from that of DMPC and DPPC (Morrow & Davis, 1987). There is some evidence from permeability studies that cholesterol must be added to DLPC bilayers to stabilize them (Hauser & Barratt, 1973; Kitagawa et al., 1976). There was no evidence in our surface density determinations that DLPC deviated from the trends in DMPC

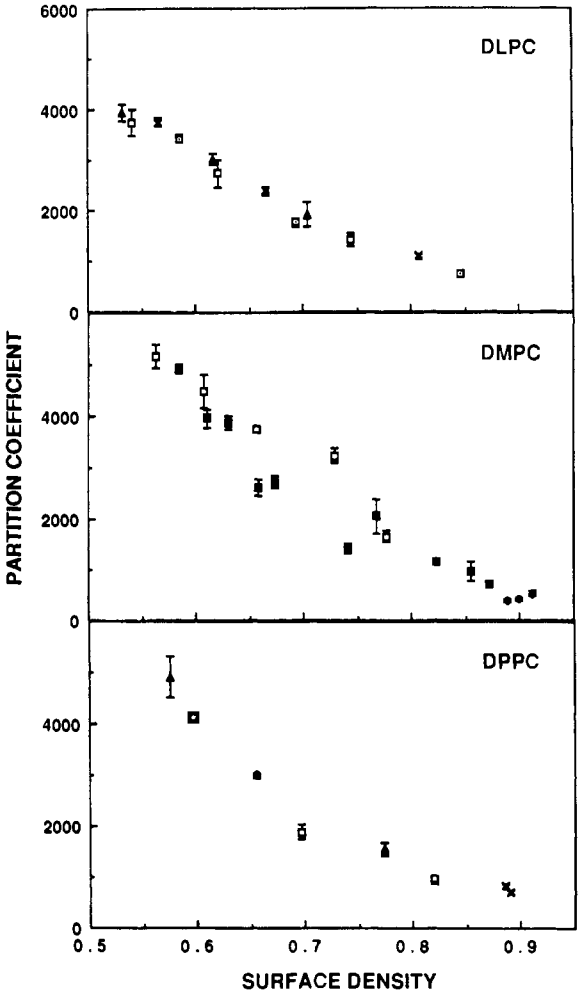

FIGURE 7: Effect of surface density on the membrane/water partition coefficient of benzene: DLPC at 20, 30, 45, and 55 °C; DMPC at 10, 25, 35, and 45 °C; DPPC at 30, 45, and 55 °C. Symbols are the same as those used in Figures 3 and 6. Benzene partitioning decreases with increasing surface density for each of the three phospholipids. In DMPC and DPPC the trend is similar; K is smaller in DLPC at low surface densities. The dependence of K on surface density is the same irrespective of whether temperature or cholesterol is used as the ordering agent.

or DPPC, even at low cholesterol concentrations. The calculated ΔH and ΔS of transfer for benzene partitioning into DLPC (data not shown) do deviate from DMPC and DPPC, but only at 20% cholesterol. It is important to notice that although the partitioning of benzene into DLPC may differ from that into DMPC and DPPC at low surface density, over most of the surface density range, from $\sigma = 0.65$ to $\sigma = 0.91$, data for all three lipids are superimposable. Therefore, the dependence of the partition coefficient on surface density appears to be a general physical property of the chain organization, independent of whether the ordering agent is cholesterol, temperature, or chain length.

CONCLUSIONS

The partitioning of solutes into bilayer membranes is often assumed to resemble the partitioning into a simple bulk oil environment; that is, the membrane/water partition coefficient is proportional to the oil/water partition coefficient. This approach takes into consideration only the chemical interactions of the solute with the bilayer hydrocarbon interior. We have demonstrated that this view neglects a second important consideration, that bilayers are interfacial, not bulk, phases of matter. Properties of interfacial phases depend on the

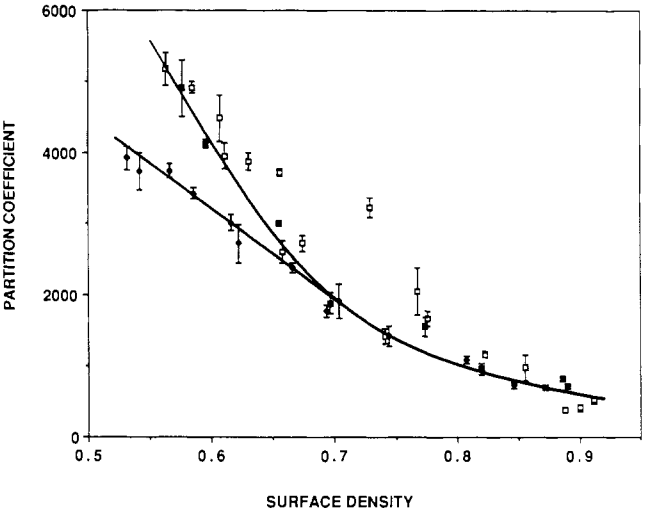

FIGURE 8: Composite of the data in Figure 7. Dependence of membrane/water partitioning on phospholipid surface density: (◆) DLPC; (□) DMPC, and (■) DPPC. It is clear that the relevant physical variable is surface density and not cholesterol concentration, temperature, or phospholipid chain length individually. Curves are hand-drawn best fits meant only to guide the eye.

surface density of the chains; in infinite bulk phases surface density plays no role. In particular, increased surface density leads to increased alignment of the phospholipid chains normal to the plane of the bilayer. One consequence is the predicted entropic expulsion of solute with increasing surface density. We have varied the surface density of the phospholipid molecules through changes in chain length, temperature, and cholesterol incorporation. We have measured the resultant surface densities using a simple technique based on ^2H NMR quadrupolar splittings. Surface densities determined in this manner are comparable to those determined by X-ray diffraction. Using a simple gas-phase equilibration method for radiolabeled benzene as solute, we have measured partitioning as a function of the surface density of the phospholipid chains. We observe that (i) solute partitioning decreases over an order of magnitude as the reduced surface density increases from 0.5 to 0.9 and that (ii) the dependence of partitioning on surface density is a general physical property of the system, irrespective of the ordering agent. These observations are at least in qualitative agreement with the principal predictions of recent statistical thermodynamic theory (Marqusee & Dill, 1986) for solute partitioning into interfacial phases of chain molecules.

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