J. Membrane Biol. 148, 157–167 (1995)

The Journal of
Membrane
Biology
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Phospholipid Surface Density Determines the Partitioning and Permeability of Acetic Acid in DMPC:Cholesterol Bilayers

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Received: 2 September/Revised: 19 July 1995

Abstract. Relationships between the permeability coefficient (P_{HA}) and partition coefficient ($K_{m/w}$) of acetic acid and the surface density of DMPC:cholesterol bilayers have been investigated. Permeability coefficients were measured in large unilamellar vesicles by NMR line broadening. Bilayer surface density, σ, was varied over a range of 0.5–0.9 by changing cholesterol concentration and temperature. The temperature dependence of P_{HA} for acetic acid exhibits Arrhenius behavior with an average apparent activation energy (E_a) of 22 ± 3 kcal/mole over a cholesterol mole fraction range of 0.00–0.40. This value is much greater than the enthalpy change for acetic acid partitioning between bulk decane and water ($\Delta H° = 4.8 \pm 0.8$ kcal/mole) and the calculated E_a (= 8.0 kcal/mole) assuming a "bulk phase" permeability model which includes the enthalpy of transfer from water to decane and the temperature dependence of acetic acid's diffusion coefficient in decane. These results suggest that dehydration, previously considered to be a dominant component, is a minor factor in determining E_a. Values of ln P_{HA} decrease linearly with the normalized phospholipid surface density with a slope of $\kappa = -12.4 \pm 1.1$ ($r = 0.90$). Correction of P_{HA} for those temperature effects considered to be independent of lipid chain order (i.e., enthalpy of transfer from water to decane and activation energy for diffusion in bulk hydrocarbon) yielded an improved correlation ($\kappa = -11.7 \pm 0.5$ ($r = 0.96$)). The temperature dependence of $K_{m/w}$ is substantially smaller than that for P_{HA} and dependent on cholesterol composition. Values of ln $K_{m/w}$ decrease linearly with the surface density with a slope of $\kappa = -4.6 \pm 0.3$ ($r = 0.95$), which is 2.7-fold smaller than the slope of the plot of ln P_{HA} *vs.* σ. Thus, chain ordering is a major determinant for molecular partitioning into and transport across lipid bilayers, regardless of whether it is varied by lipid composition or temperature.

Introduction

From permeability experiments dating back to Overton's rules formulated nearly a century ago (Overton, 1899) scientists have observed that permeation rates through biological and artificial membranes often depend on the chemical character of the permeant in a manner similar to the oil/water partition coefficient (Diamond et al., 1974; Finkelstein, 1976; Orbach & Finkelstein, 1980; Walter & Gutknecht, 1986). From these observations evolved the general assumption that solute partitioning from water into and diffusion through the membrane lipid region resembles that which would occur within a "homogeneous slab" of bulk solvent such as olive oil, octanol, or hydrocarbon. This "bulk phase" model, however, fails to consider interfacial constraints that lead to chain-order gradients in the bilayer interior (Fenske & Cullis, 1993; Seelig & Seelig, 1974), and therefore is unable to account for a large number of observations regarding molecular permeability in lipid bilayer membranes. For example, diffusion coefficients calculated from permeabilities are often orders-of-magnitude smaller than those obtained in bulk solvents. Secondly, solute permeation shows a much steeper dependence on permeant size than diffusion in bulk solvents (Lieb & Stein, 1986; Xiang & Anderson, 1994*b*). Thirdly, membranes which are known to be more "ordered" as a result of polar head group composition or due to increased cholesterol concentrations (Finkelstein, 1976; Todd, Melhorn & Macey, 1989*a,b;* Sada et al., 1990) or at lower temperatures (Brahm, 1983; Magin & Niesman, 1984; Bar-On & Degani, 1985), or monolayers under high lateral pressures (Peters & Beck, 1983) have greater resistances to permeation often by orders-of-magnitude.

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Fourthly, there exist strong enthalpy-entropy compensations and large Barclay-Butler constants in bilayer/water partitioning (Beschiaschvili & Seelig, 1992; Katz & Diamond, 1974*b*) and membrane/water partition coefficients decrease significantly with increasing lipid surface density (DeYoung & Dill, 1990). Finally, solutes distribute nonuniformly within bilayers (Marqusee & Dill, 1986; Subcznski, Hyne & Kusuml, 1991; Xiang & Anderson, 1994*a*). Small, hydrophobic molecules such as low molecular weight alkanes have been shown by ^{2}H-NMR, low angle X-ray diffraction, and neutron diffraction (McIntosh, Simon & MacDonald, 1980; White, King & Cain, 1981; Pope, Walker & Dubro, 1984) to localize in the center of bilayers. Polar solutes, such as alkanols, on the other hand, reside in the peripheral region of bilayers with the hydroxyl group anchored at the bilayer-aqueous interface (Miller, Hammond & Porter, 1977; Pope et al., 1984). Windrem and Plachy (1980) have shown that the product of solubility and diffusion coefficients for oxygen in phospholipid bilayers depends strongly on the location at which oxygen interacts with a spin label attached to the chain backbone. Although there is now abundant evidence to indicate that membrane models based on bulk solvent analogies are inadequate to explain lipid bilayer uptake and transport, no systematic studies have yet been performed to account for the interfacial, highly ordered nature of bilayers.

This work represents the first attempt to define quantitatively the relationships between solute partition and permeability coefficients and the physical states of the lipid bilayers as characterized by local chain ordering and surface density. Inasmuch as the primary function of biological membranes is to control uptake and permeation of solutes, such an investigation is essential to a full understanding of cell function and the biological barrier to drug delivery. The main objective of this study is to examine the central hypothesis that chain ordering in lipid bilayers is a major determinant for molecular transport across lipid bilayers. We have explored the partitioning and permeation of acetic acid in dimyristoylphosphatidylcholine (DMPC)/cholesterol lipid bilayers varying in cholesterol concentration and at various temperatures to characterize the relationship between these processes and the phospholipid surface density, which is quantitatively correlated with order parameters in the highly-ordered acyl chain region of lipid bilayers (DeYoung & Dill, 1988; Nagle, 1993).

Acetic acid's bilayer permeability coefficients were measured in large unilamellar vesicles (LUVs) using an NMR line-broadening method first developed by Alger and Prestegard (1979). The characterization of these LUVs and validation studies to explore the influence of permeant concentration, chemical shift reagent concentration, pD, ionic strength, vesicle size distribution, and different extrusion procedures for LUV preparation on the permeability data obtained by NMR line broadening have been reported elsewhere (Xiang & Anderson, 1995*a*). Compared with other transport techniques, the present method has the following promising features: (i) since permeants inside and outside the vesicle are distinguishable spectroscopically, no separation of entrapped permeant is required and thus, fluxes in the time frame of 1 msec $\rightarrow$ 1 sec, within which the permeation lifetimes of a large number of simple molecules reside, can be obtained; (ii) there is no net flux of chemically distinguishable species and the system is in true thermodynamic equilibrium, in contrast to physical separation methods and studies using planar lipid bilayers (Xiang, Chen & Anderson, 1992; Xiang & Anderson, 1994*b*); and (iii) aqueous boundary layer effects are negligible (Xiang & Anderson, 1995*a*).

Materials and Methods

Materials

DMPC was purchased from Avanti Polar Lipids (Pelham, AL). Cholesterol (99+%) was purchased from Sigma (St. Louis, MO). Acetic acid (99.8%) was purchased from Aldrich (Milwaukee, WI). Radiolabeled ^{3}H-acetic acid and ^{14}C-glycerol were obtained from ICN Biomedicals (Irvine, CA) and New England Nuclear (Boston, MA), respectively. Phospholipid purity was checked by thin-layer chromatography. All other reagents were obtained commercially and were of analytical reagent grade. Polycarbonate membranes and membrane holders were obtained from Nuclepore (Pleasanton, CA).

LUV Liposome Preparation

LUVs were prepared by a modified combined technique due to Bangham, Standish & Watkins (1965) and Olson et al. (1979). DMPC (7–10 mg) and cholesterol (0–3 mg) were accurately weighed, dissolved in chloroform, rotary evaporated to a dry thin film on the bottom of a 50 ml round-bottom flask at ≈40°C, and left under vacuum for 2 hr at ≈50°C. An aqueous solution (≈1 ml) containing 30–50 mM acetic acid was then added and the lipids were hydrated by repeating 10 times the procedure of vortexing for 30 sec and slow shaking for 30 sec, followed by gentle shaking for 30 min and incubation overnight at 30°C. The multilamellar vesicles (MLVs) formed were forced through a 0.1 μm polycarbonate membrane filter 17 times before the NMR transport experiments.

Determination of Permeability Coefficients across Lipid Bilayers

The apparent permeability coefficients for acetic acid across the DMPC:cholesterol bilayers were determined as a function of pD (5–7), cholesterol composition (X_{chol} = 0.0–0.4) and temperature (21–50°C) by the ^{1}H-NMR line-broadening method developed by Alger and Prestegard (1979) and further validated in a recent study by the authors (Xiang & Anderson, 1995*a*). LUVs containing 30–50 mM acetic acid were prepared in deuterated water as described above. The experiments were performed on the Bruker-200 NMR spectrometer operated in the Fourier transform mode at 200 MHz. Samples were equilibrated

for 20 min at a given temperature controlled by a standard variable temperature accessory (BVT1000, Bruker). Each spectrum was the average of 32–1000 acquisitions separated by 3–7-sec pulse delays. The spectra were Fourier transformed and phased with an ASPECT 3000 computer. The resonance frequencies of the methyl protons of acetic acid located inside and outside the vesicles, ω_i and ω_o, were separated by adding an impermeable shift reagent, $Pr(NO_3)_3$ (final concentration, 5 mM), to the sample before the spectral acquisitions.

The lifetime of the permeant inside the vesicle, τ_i, was obtained using the following line-width expression in the slow exchange limit, $|\omega_i - \omega_o|T_{2,i} >> 1$, (Piette & Anderson, 1959)

$$\pi\Delta\nu = 1/T_{2,i} + 1/\tau_i \tag{1}$$

where $\Delta\nu$ is the full line width at one-half the maximum peak height and $T_{2,i}$ is the spin-spin relaxation time which includes heterogeneous line broadening in the absence of exchange. The line-width in the absence of exchange ($1/T_{2,i}$ = 2.6–3.4 Hz) was obtained at a low temperature (10°C) where the permeation rate is negligible. The apparent permeability coefficient (P_{app}) is equal to $V/(A\tau_i)$, where V is the entrapped volume and A is the surface area. The V/A ratio was determined from hydrodynamic diameter d as obtained in our dynamic light scattering (DLS) measurements according to the formula

$$V/A = (d - \Delta r)/6 \tag{2}$$

where Δr is the bilayer thickness. The bilayer thickness is 3.6 nm for cholesterol-free DMPC bilayers at 27°C (Rand & Parsegian, 1989). For DMPC membranes containing different cholesterol concentrations and at different temperatures, the relation $\Delta r = C/A_{DMPC}$ is used, where C is a constant and A_{DMPC} is the surface area of the phospholipid. In the DLS experiments, the apparatus consisted of a photon correlation spectrometer (Model BI-90, Brookhaven Instruments, Holtsville, NY) and a He-Ne laser light source at 632.8 nm wavelength. One drop of LUV suspension was placed in a 13 × 75 mm cleaned glass test tube and brought to a volume of 2 ml with the same filtered solution in the presence of 5 mM Pr^{3+} used to prepare the LUVs. The sample was put in a temperature-controlled cuvette holder with a toluene index-matching bath. Autocorrelation functions were determined for a period of 100 sec with a 10–80 μsec duration at 90° and analyzed by the method of cumulants. DLS measurements indicated no significant changes (<6%) in the mean vesicle size over a period of 3 days.

Determination of Membrane/Water and Decane/Water Partition Coefficients

DMPC:cholesterol membrane/water partition coefficients for acetic acid were determined at several cholesterol concentrations (X_{chol} = 0.0–0.4) and temperatures (25–49°C) using a radiotracer method. Briefly, 3 mM acetic acid solution at an ionic strength of 0.1 was spiked with ^{3}H-labeled acetic acid and ^{14}C-glycerol. An aliquot (3 ml) of this solution was then added to ≈60 mg dried lipid film to form a suspension of MLVs using the method described above. After an equilibration time of 8 hr at a controlled temperature, the MLVs were separated from the aqueous phase by ultracentrifugation at 25,000 × g for 20 min in a Sorvall RC-2B centrifuge (DuPont, Wilmington, DE) at the same temperature. The supernate and the liposome precipitate were removed and transferred to scintillation vials for scintillation counting. The calculation of the molal partition coefficient, $K_{m/w}$, required correction for bulk water and nonsolvent water within the liposome precipitate, as described elsewhere (Xiang & Anderson, 1995*a*). ^{14}C-glycerol was used to determine the total weight of water in the liposome precipitate, while literature values for the fraction of nonsolvent water was used (Katz & Diamond, 1974*a*), assuming that the nonsolvent water depends only on the total amount of DMPC.

Decane/water partition coefficients for acetic acid were measured as a function of temperature (25–50°C) using the shake flask method at a pH well below the pK_a of acetic acid to ensure that >99% was in its unionized form and at concentrations such that acetic acid was predominantly monomeric in both phases (i.e., $[HA]_{aq}$ < 10 mM). The partition coefficients were calculated as the ratios between the molar concentrations in hydrocarbon and water, determined by HPLC as described in a separate publication (Xiang & Anderson, 1995*a*).

Results and Discussion

Determination of Permeability Coefficients—pD Effects

Repeated extrusion of large multilamellar vesicles through a 0.1 μm filter has been shown by ^{31}P-NMR to yield primarily unilamellar vesicles (Hope et al., 1985). The liposome-specific aqueous volume, V/A, needed to determine permeability coefficients from the NMR line broadening, was calculated from the hydrodynamic diameter. Mean vesicle diameters and polydispersities obtained from our DLS measurements at different temperature and mole fractions of cholesterol are presented in Table 1. The hydrodynamic diameter increases slightly with temperature, which may be caused by changes of the lipid surface area with temperature.

To explore the barrier properties of lipid bilayers varying widely in composition and temperature by the NMR line-broadening technique, one needs to select an ionizable permeant so that, by adjusting pD, suitable line-widths can be selected even when membrane resistance to transport may vary by several log units. Upon the addition of 5 mM Pr^{+3} into a vesicle suspension containing acetic acid, the extravesicular proton peak is shifted from ≈1.8 ppm to ≈3.0 ppm while the internal resonance line width is broadened to an extent that depends on pD, cholesterol composition, and temperature (Xiang & Anderson, 1995*a*). The apparent permeability coefficient obtained from line broadening ($P_{app} = V/(A\tau_i)$, *see* Eq. 1) can be written as

$$P_{app} = \frac{V}{\tau_i A} = f_{HA}P_{HA} + (1 - f_{HA})P_A \tag{3}$$

where P_{HA} and P_A are the permeability coefficients for the neutral and ionized species, respectively. f_{HA} is the fraction of acetic acid existing in neutral form, $f_{HA} = 1/(1 + K_a/[D^+])$, where $K_a = 7.55 \times 10^{-6}$ is the dissociation constant for acetic acid in deuterated water (Korman & LaMer, 1936). The pD value was obtained by adding 0.4 units to the pH meter reading (Glasoe & Long, 1960). Representative profiles of P_{app} at 303 K *vs.* f_{HA} at three

Table 1. Acetic acid's permeability coefficients across DMPC:cholesterol LUVs along with the hydrodynamic diameter d and polydispersity as a function of temperature and cholesterol concentrations

X_{chol}	T (°C)	σ^a	d (nm)[c]	Polydispersity[c]	P_m (cm/sec)[d]
0.00	26	0.6129	128 ± 5[b]	0.12 ± 0.02[b]	$(6.5 \pm 0.6) \times 10^{-3}$ [b]
	30	0.6043	137 ± 5	0.13 ± 0.04	$(1.2 \pm 0.4) \times 10^{-2}$
	33	0.5945	135 ± 3	0.12 ± 0.03	$(1.3 \pm 0.3) \times 10^{-2}$
	36	0.5858	135 ± 5	0.12 ± 0.02	$(2.0 \pm 0.4) \times 10^{-2}$
0.10	21	0.6660	120 ± 5	0.05 ± 0.03	$(2.7 \pm 0.5) \times 10^{-3}$
	26	0.6562	128 ± 3	0.07 ± 0.01	$(3.6 \pm 0.3) \times 10^{-3}$
	30	0.6484	127 ± 5	0.16 ± 0.02	$(7.5 \pm 0.4) \times 10^{-3}$
	40	0.6172	132 ± 6	0.10 ± 0.02	$(1.6 \pm 0.3) \times 10^{-2}$
0.20	21	0.7866	124 ± 3	0.07 ± 0.05	$(9.1 \pm 0.8) \times 10^{-4}$
	26	0.7350	124 ± 5	0.10 ± 0.02	$(1.4 \pm 0.1) \times 10^{-3}$
	30	0.7000	133 ± 3	0.12 ± 0.01	$(2.4 \pm 0.2) \times 10^{-3}$
	35	0.6779	132 ± 4	0.07 ± 0.01	$(4.2 \pm 0.4) \times 10^{-3}$
	40	0.6603	142 ± 5	0.10 ± 0.02	$(9.4 \pm 1.0) \times 10^{-3}$
0.30	21	0.8685	121 ± 8	0.05 ± 0.03	$(2.9 \pm 0.4) \times 10^{-4}$
	25	0.8549	124 ± 5	0.06 ± 0.04	$(4.6 \pm 0.4) \times 10^{-4}$
	28	0.8318	126 ± 5	0.08 ± 0.03	$(1.1 \pm 0.1) \times 10^{-3}$
	30	0.8164	127 ± 3	0.10 ± 0.02	$(1.3 \pm 0.1) \times 10^{-3}$
	37	0.7599	126 ± 6	0.05 ± 0.02	$(2.4 \pm 0.2) \times 10^{-3}$
	39	0.7535	139 ± 4	0.04 ± 0.03	$(3.5 \pm 0.3) \times 10^{-3}$
	50	0.7103	141 ± 5	0.12 ± 0.03	$(1.7 \pm 0.3) \times 10^{-2}$
0.40	25	0.8746	133 ± 7	0.05 ± 0.03	$(2.5 \pm 0.3) \times 10^{-4}$
	30	0.8549	137 ± 6	0.08 ± 0.05	$(4.8 \pm 0.4) \times 10^{-4}$
	35	0.8287	137 ± 5	0.06 ± 0.03	$(8.3 \pm 0.5) \times 10^{-4}$
	40	0.7971	144 ± 3	0.10 ± 0.03	$(1.5 \pm 0.1) \times 10^{-3}$
	50	0.7555	144 ± 5	0.10 ± 0.02	$(7.0 \pm 0.5) \times 10^{-3}$

[a] Normalized surface density (DeYoung & Dill, 1988). [b]Expressed as mean ± SD. [c]The number of DLS measurements = 3–4. [d]The standard deviation is a propagated error of both the DLS measurement and the NMR line-width determination.

different mole fractions of cholesterol (X_{chol}) in DMPC bilayers are shown in Fig. 1. Linear regression analyses yielded permeability coefficients, P_{HA}, of $(1.2 + 0.1) \times 10^{-2}$ cm/sec, $(2.4 \pm 0.3) \times 10^{-3}$ cm/sec, and $(5.2 \pm 0.2) \times 10^{-4}$ cm/sec for $X_{chol} = 0.0$, 0.20, and 0.40, respectively. The apparent permeability coefficients approach zero at high pD within the intercept uncertainty ($\pm 9 \times 10^{-6}$ cm/sec), suggesting that the permeability of the ionized species is negligible. The linearity of these plots suggests that variation of pD in the range of 5–7 does not significantly alter bilayer barrier properties. This is consistent with previous experiments in these laboratories on planar lipid bilayers (Xiang et al., 1992) in which the permeability of acetamide was found to be independent of solution pH over a wider pH range.

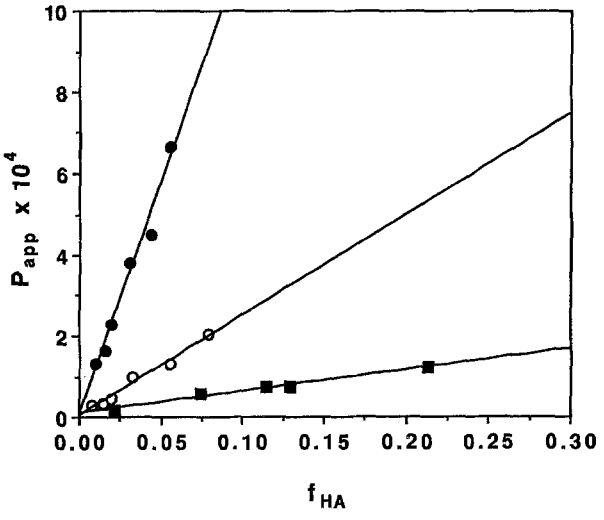

Fig. 1. The apparent permeability coefficient for acetic acid in LUVs at 303 *K vs.* the fraction of acetic acid existing in neutral form, $f_{HA} = 1/(1 + K_a/[D^+])$: ●: $X_{chol} = 0.00$; ○: $X_{chol} = 0.20$; and ■: $X_{chol} = 0.40$. X_{chol} is the mole fraction of cholesterol in DMPC lipid bilayer vesicles.

Cholesterol and Temperature Dependence of Permeability Coefficients

The temperature dependence of the permeability coefficient for acetic acid is shown in Fig. 2 and Table 1 at several cholesterol concentrations. The permeability coefficient increases with temperature and decreases with the cholesterol concentration, varying from 2.0×10^{-2} cm/sec at 309 K and $X_{chol} = 0.00$ to 2.5×10^{-4} cm/sec at 298 K and $X_{chol} = 0.40$. Cholesterol significantly increases the orientational and conformational order of the phospholipid chains in DMPC/cholesterol bilayers above their gel-to-liquid-crystalline phase transition, leading to speculation that this might be responsible for the reduced

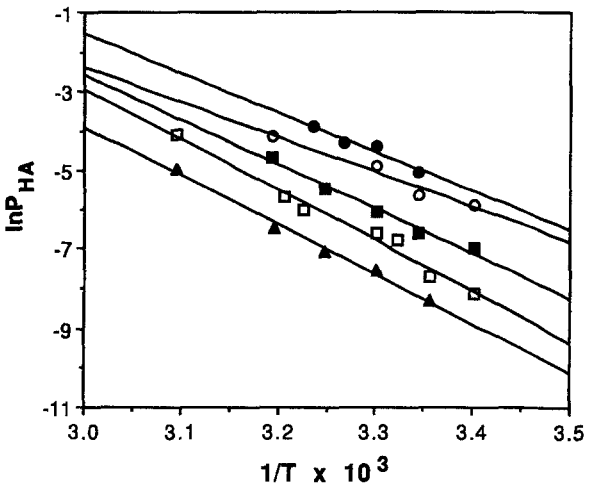

Fig. 2. Arrhenius plots of the permeability coefficient for acetic acid at different mole fractions of cholesterol in the DMPC lipid bilayers: ●: $X_{chol} = 0.00$; ○: $X_{chol} = 0.10$; ■: $X_{chol} = 0.20$; □: $X_{chol} = 0.30$; ▲: $X_{chol} = 0.40$.

permeation of small molecules in cholesterol-containing bilayers (Weisz et al., 1992). A quantitative evaluation of cholesterol's effect on permeability has been difficult, however, due to several factors. First, in transport experiments using planar lipid bilayer films, the mole fraction of cholesterol in the bilayer formed may differ from that in the applied liquid suspension due to partitioning of cholesterol between the bilayer and the surrounding annular region (Huang, Wheeldon & Thompson, 1964; Redwood & Haydon, 1969). There is no direct and quantitative way to estimate the resulting difference in the cholesterol concentration. Second, in most transport experiments conducted previously using liposome preparations, the vesicles formed were either multilamellar or small unilamellar (sonicated) with a broad size distribution. In transport studies using MLVs, only amounts of released permeants per unit time can be measured, from which one is still unable to calculate the permeability coefficient across the lipid bilayers. In transport studies using mixed lipid SUVs, the cholesterol may not be uniformly distributed between the inner and outer monolayers because of the high curvature of the lipid bilayers (Huang et al., 1974; Newman & Huang, 1975).

The apparent activation energies, E_a, for the permeability of acetic acid across liquid crystalline DMPC: cholesterol lipid bilayers were obtained by fitting the data in Fig. 2 above the main transition temperature. The results are listed in Table 2. High apparent activation energies (18–26 kcal/mol) were obtained at all cholesterol compositions employed. Increasing cholesterol content in the bilayers increases E_a from 20 kcal/mol at $X_{chol} = 0.00$ to 25 kcal/mol at $X_{chol} = 0.40$. These values are substantially higher than the E_a reported for acetic acid transport through egg PC/phosphatidic acid bilayers (10 ± 4 kcal/mol) by Alger and Prestegard (1979). This may be due to the relative insensitivity of the chain ordering, as characterized by the normalized surface density, σ, to temperature in egg-PC bilayers ($\Delta\sigma/\sigma\Delta T$) = 0.28%°C^{-1} (Stockton et al., 1976), as compared to DMPC:cholesterol bilayers ($\Delta\sigma/\sigma\Delta T$) = 0.45%, 0.40%, 0.74%, 0.92% and 0.59%°C^{-1}, respectively, for X_{chol} = 0.0, 0.1, 0.2, 0.3, and 0.4 (DeYoung & Dill, 1988)). Activation energies within a similar range have been reported for the transport of n-alkylamines across egg-lecithin bilayers (≈20 kcal/mol, (Bar-On & Degani, 1985)), for glucose from DMPC/DCP vesicles (26 kcal/mol, (Bresseleers, Goderis & Tobbach, 1984)), and for polyhydroxyalcohols through DMPC/30% cholesterol membranes (14–25 kcal/mol, (de Gier et al., 1971)).

Table 2. Apparent activation energies and enthalpies of transfer calculated from the temperature dependence of the permeability and partition coefficients, respectively, of acetic acid in DMPC:cholesterol lipid bilayers

X_{chol}	E_a (kcal/mol)	$(\Delta H°)_{w->m}$ (kcal/mol)
0.00	20 ± 3[a]	2.5 ± 0.5[a]
0.10	18 ± 2	5.3 ± 0.6
0.20	22 ± 2	7.2 ± 0.7
0.30	26 ± 2	5.2 ± 0.5
0.40	25 ± 2	5.5 ± 0.7
bulk	8.0[b]	4.8 ± 0.8[c]

[a] Expressed as mean ± SD.
[b] Predicted from the measured decane/water partition coefficients and the published data on diffusion and viscosity in decane.
[c] From decane/water partition coefficients.

A consideration of the components of the activation energy leads to the conclusion that it is the chain ordering within the bilayer and its variation with temperature that is primarily responsible for the large values observed. The activation energy may be considered in terms of the following contributions: (i) dehydration of the permeant molecule accompanying its entry from water into the bilayer interior assuming a "bulk phase" model of the interior; (ii) variation in the diffusion coefficient with temperature within the barrier domain assuming a "bulk phase" membrane model; and (iii) the additional effects of temperature induced changes in local chain packing structure in the barrier region, usually characterized by segmental order parameters, on solute permeability.

The dehydration step has previously been suggested to be a dominant activation process for solute transport across lipid bilayers and other biological membranes (de Gier, et al., 1971; Bindslev & Wright, 1976; Stein, 1986). To test this hypothesis, the partition coefficient for acetic acid between bulk decane and water as a function of temperature was determined as shown in Fig. 3 (Recent transport studies conducted in these laboratories demonstrated that the transport barrier within egg lecithin bilayers resembles the partially unsaturated hydrocarbon 1,9-decadiene more closely than a fully saturated

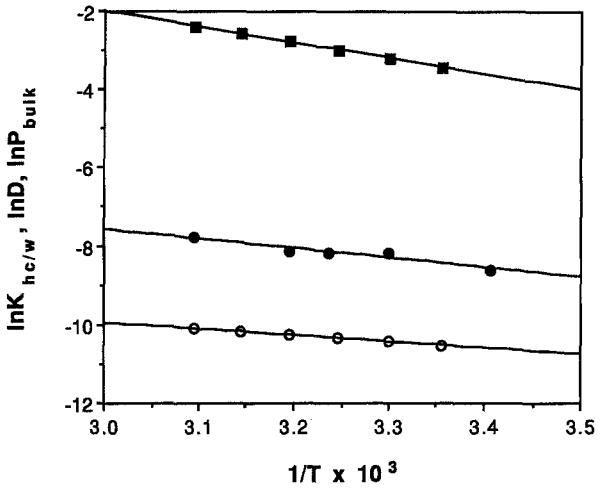

Fig. 3. Arrhenius plots of acetic acid's decane/water partition coefficient (●), $K_{w/hc}$, diffusion coefficient in decane (cm^2/sec) (○), D, and "bulk phase" permeability coefficient (■), $P = K_{w/hc}\, D/d$ (cm/sec), where $d = 18$ Å.

hydrocarbon (Xiang & Anderson, 1994*b*; Xiang & Anderson, 1994*c*; Xiang, et al., 1992). However, decane was chosen for the present comparison because the alkyl chains in DMPC are fully saturated). The molar enthalpy change for partitioning of acetic acid from water to decane, obtained from a least-squares fit of the data in Fig. 3, is $\Delta H° = 4.8 \pm 0.8$ kcal/mole, much smaller than the apparent activation energy for permeability across DMPC:cholesterol lipid bilayers. The dehydration process is therefore only a minor component of the overall activation energy.

If permeation is rate-limited by a distinct barrier domain within the bilayer, the permeability coefficient, P_{HA}, is approximately proportional to the product of the partition coefficient between this barrier domain and water, $K_{barrier/w}$, and the diffusion coefficient within the barrier domain, $D_{barrier}$,

$$P_{HA} = \frac{K_{barrier/w} D_{barrier}}{\delta_{barrier}} \tag{4}$$

where $\delta_{barrier}$ is the thickness of the barrier domain. In the absence of chain ordering effects, diffusion in the barrier domain would be expected to resemble that in a bulk hydrocarbon solvent. A large quantity of data suggest that diffusion coefficients in bulk solvents are proportional to temperature and the reciprocal of viscosity of the organic solvent, η (Scheibel, 1954). Using an approach described previously to estimate the bulk diffusion coefficient of acetic acid (Xiang & Anderson, 1994*b*) and compiled viscosity-temperature data, the diffusion coefficient for acetic acid as a function of temperature was calculated, as plotted in Fig. 3. Assuming the effective thickness of the barrier domain to be about 70% of that of the overall hydrocarbon chain region, or roughly 18Å, the "bulk phase" model permeability coefficient for acetic acid, P_{bulk}, may be predicted. These results are also presented in Fig. 3. Thus, if the bilayer barrier domain behaves like a simple hydrocarbon liquid, the observed permeability coefficient should follow closely the Arrhenius curve (solid squares) shown in Fig. 3, which yields an apparent activation energy of 8.0 kcal/mole, still much smaller than that for the observed permeabilities across lipid bilayers. Thus, the additional contribution due to chain ordering within the bilayer membrane and its variation with temperature is primarily responsible for the large apparent activation energy observed. The strong dependence of the permeability coefficient on cholesterol concentration and the apparent increases in E_a with increasing cholesterol composition also support this conclusion.

Cholesterol and Temperature Dependence of Partition Coefficients

Membrane/water partition coefficients for acetic acid, $K_{m/w}$, were measured at several cholesterol concentrations and temperatures. The results are presented in Fig. 4 and Table 3. The partition coefficient increases with temperature and decreases with the cholesterol concentration, varying from 1.63 ± 0.08 at 322 K and $X_{chol} = 0.0$ to 0.33 ± 0.02 at 298 K and $X_{chol} = 0.4$. This ≈5-fold variation in the value of the partition coefficient contrasts markedly with the >70-fold variation in permeability coefficients over the same range of temperature and cholesterol composition. The dependence of acetic acid's $K_{m/w}$ on cholesterol concentration is in qualitative agreement with the partitioning data from a number of studies in DMPC/cholesterol membranes (Antunes-Madeira & Madeira, 1984; Antunes-Madeira & Madeira, 1985; DeYoung & Dill, 1988; DeYoung & Dill, 1990) but differs from that found for partitioning of long-chain fatty acids (N_{carbon} = 10–16) into bilayers, which is independent of the mole fraction of cholesterol in the membranes (Peitzsch & McLaughlin, 1993). Linear least-squares fits of the data in Fig. 4 gave the slopes and therefore the standard molal enthalpies of transfer, $(\Delta H°)_{w->m}$, (van't Hoff relation) as a function of cholesterol composition as listed in Table 2.

A strong enthalpy-entropy compensation was found (*not shown*) in DMPC:cholesterol bilayers varying in cholesterol composition, such that increases in enthalpy of transfer were largely compensated by corresponding increases in the entropy of transfer, leading to only modest changes in partition coefficients with X_{chol}. Strong enthalpy-entropy compensation has also been observed in recent studies on binding of peptides and hydrophobic anions and cations of quite different chemical structure (Seelig & Ganz, 1991; Beschiaschvili & Seelig, 1992) where the state of bilayer chain packing was varied by

Table 3. Acetic acid's partition coefficients between DMPC:cholesterol membranes and water at different temperature and cholesterol concentrations

X_{chol}	T (°C)	σ^b	$K_{m/w}{}^a$
	30	0.6043	1.3 ± 0.1
	35	0.5879	1.49 ± 0.06
	40	0.5775	1.5 ± 0.1
	45	0.5660	1.5 ± 0.1
	49	0.5568	1.63 ± 0.08
0.10	20	0.6680	(6.6 ± 0.4) × 10^{-1}
	25	0.6582	(6.9 ± 0.5) × 10^{-1}
	30	0.6484	(8.9 ± 0.3) × 10^{-1}
	35	0.6275	(9.99 ± 0.07) × 10^{-1}
	40	0.6172	1.01 ± 0.07
	45	0.6066	1.45 ± 0.04
	49	0.5961	1.38 ± 0.01
0.20	25	0.7438	(4.8 ± 0.7) × 10^{-1}
	30	0.7000	(6.3 ± 0.6) × 10^{-1}
	35	0.6779	(7.1 ± 0.9) × 10^{-1}
	40	0.6603	(9.6 ± 0.4) × 10^{-1}
	45	0.6603	1.13 ± 0.00
	49	0.6425	1.1 ± 0.2
0.30	25	0.8549	(3.7 ± 0.4) × 10^{-1}
	30	0.8164	(4.5 ± 0.7) × 10^{-1}
	35	0.7705	(5.5 ± 0.3) × 10^{-1}
	40	0.7492	(6.1 ± 0.2) × 10^{-1}
	45	0.7314	(6.6 ± 0.5) × 10^{-1}
	49	0.7145	(7.3 ± 0.2) × 10^{-1}
0.40	25	0.8746	(3.3 ± 0.2) × 10^{-1}
	30	0.8549	(4.5 ± 0.4) × 10^{-1}
	35	0.8287	(5.1 ± 0.3) × 10^{-1}
	40	0.7971	(5.4 ± 0.4) × 10^{-1}
	45	0.7783	(6.1 ± 0.2) × 10^{-1}
	49	0.7601	(7.3 ± 0.6) × 10^{-1}

[a] Expressed as mean ± SD.
[b] Normalized surface density from DeYoung and Dill (1988).

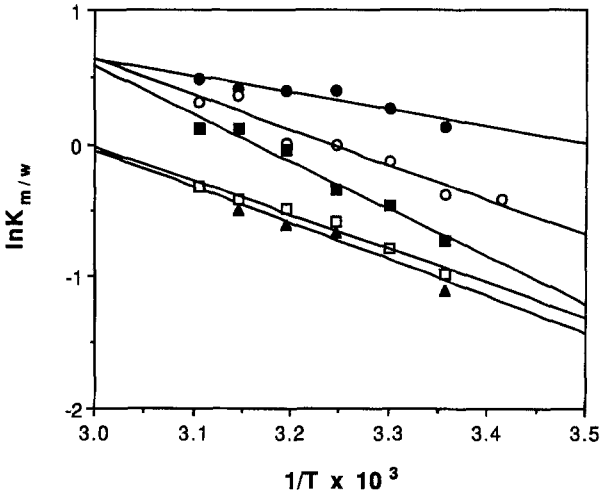

Fig. 4. Arrhenius plots of the membrane/water partition coefficient for acetic acid at different mole fractions of cholesterol in the DMPC lipid bilayers: ●: X_{chol} = 0.00; ○: X_{chol} = 0.10; ■: X_{chol} = 0.20; □: X_{chol} = 0.30; and ▲: X_{chol} = 0.40.

vesicle size. In the study of the binding of a peptide drug, SMS 201-995 (Beschiaschvili & Seelig, 1992), the difference in the free energy of binding between small sonified POPC/POPG vesicles and large extruded vesicles was found to be only 0.5 kcal/mol while the enthalpy of transfer changed by 8 kcal/mol.

The transfer enthalpies listed in Table 2 are substantially smaller than the corresponding apparent activation energies. This can be largely rationalized by considering that: (i) the apparent E_a also includes temperature effects on diffusivity; and (ii) the permeability and partitioning experiments probe different domains of the bilayer. Thus, whereas previous transport studies in these laboratories have established that the transport barrier is hydrocarbonlike in its chemical selectivity (Xiang, et al., 1992; Xiang & Anderson, 1994*b*; Xiang & Anderson, 1994*c*), polar, hydrophilic solutes reside in a region close to the bilayer headgroups (Miller et al., 1977; Pope et al., 1984), the microenvironment of which can be mimicked by isoamyl alcohol (Diamond & Katz, 1974). It is thus possible that the contribution of solvation interactions to the molal enthalpy of acetic acid in water may be similar to that in the partitioned domain in the bilayers. Moreover, the interaction energy of acetic acid with DMPC molecules may be similar to that with the intercalated cholesterol molecules. This is possible if acetic acid resides in the phospholipid ester region where it can form hydrogen bonds with both phospholipid ester carbonyls and cholesterol hydroxyl groups as X-ray diffraction and neutron diffraction measurements have indicated that the hydroxyl in cholesterol is in the immediate vicinity of the phospholipid ester carbonyl (Franks, 1976; Worcester & Franks, 1976).

Interpretation of Acetic Acid Permeability and Partition Coefficients in Terms of Bilayer Order

The normalized surface density, $\sigma = A_o/A_{DMPC}$, where A_{DMPC} is the area per DMPC molecule and A_o is its area in the crystal, can be obtained from the measured order parameter (S_{mol}) in the plateau region via a formula derived from lattice theory (DeYoung & Dill, 1988)

$$\sigma = \frac{1}{3}(1 + 2S_{mol}) \tag{5}$$

A new expression was derived recently by Nagle (1993) through a geometric analysis of a hydrocarbon chain in a lipid bilayer. It was claimed to be a more accurate representation of hydrocarbon chains than the lattice model, but several major approximations are also built into this model, including the assumptions that the local density is a constant independent of depth in the bilayer and that upturns of chain segments are negligible. Recent molec-

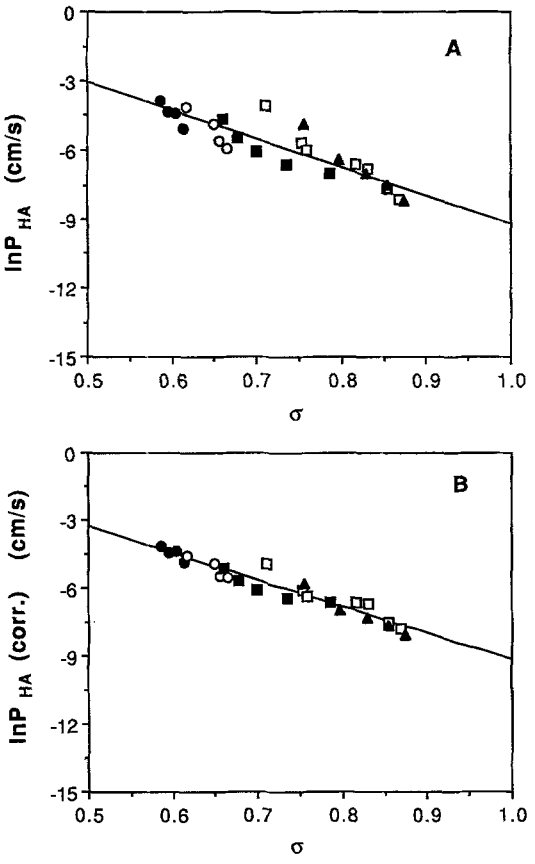

Fig. 5. Natural log of permeability coefficient (*A*) and corrected permeability coefficient according to Eq. 7 (*B*) across DMPC:cholesterol lipid bilayers *vs.* surface density σ: ●: $X_{chol} = 0.00$; ○: $X_{chol} = 0.10$; ■: $X_{chol} = 0.20$; □: $X_{chol} = 0.30$; ▲: $X_{chol} = 0.40$.

ular dynamics simulations conducted in these laboratories have shown that the local density is not a constant (Xiang, 1993) and that upturn events do occur in lipid bilayers with a low but detectable probability (Xiang & Anderson, 1995*b*). Therefore, in the following analyses, the surface density data reported by DeYoung and Dill and their linear interpolations whenever a specific surface density was not available were used. Because of the linear relationship between the order parameter and surface density in *both* models, switching to the Nagle model is straightforward and would not alter any conclusions emerging from this study. The possibility that the order parameters in LUVs may differ from those in MLVs due to variation in vesicle size was recently examined by Fenske and Cullis (1993). They found that the order-parameter profiles in POPC-d_{31} LUVs were very similar to those of MLVs at the same temperature and chemical composition, indicating that orientational order in both MLVs and LUVs with diameters $\geq$100 nm is essentially the same. Since the LUVs employed in the present work had hydrodynamic diameters between 120 nm and 144 nm, the assumption was made that order did not depend on vesicle size.

When all the permeability data measured at different temperatures and cholesterol concentrations were plotted as their logarithms *vs.* the normalized surface density (σ) in Fig. 5*A*, a clear linear relationship was obtained

$$\ln P_{HA} = \xi + \kappa\sigma \tag{6}$$

A least-squares fit of these data using Eq. 6 gave a slope of $\kappa = -12.4 \pm 1.1$ ($r = 0.90$). The scatter of the data points from the regression line was not completely random, however. One systematic trend which could be readily seen was that, at a given surface density, the permeability coefficient at a higher mole fraction of cholesterol exceeded that at a lower mole fraction of cholesterol. Since, at a constant surface density, the lipid bilayer with a higher cholesterol concentration must be at a higher temperature, the data in Fig. 5*A* were corrected for temperature effects which are independent of contributions of chain ordering in the lipid bilayers. These temperature effects may include the dehydration energy for partitioning and kinetic energy for diffusion and the volume expansion in the lipid bilayers as predicted by a bulk phase model for partitioning and diffusion (Fig. 3). Using $T_o = 303$ *K* as a reference temperature, the corrected permeability coefficient at *T* is defined as

$$P_{HA}^{corr}(T) = P_{HA}(T)e^{\Delta E_a(T_o - T)/k_B T T_o} \tag{7}$$

where $\Delta E_a = 8.0$ kcal/mol. A least-squares fit of the corrected permeability coefficients, plotted in Fig. 5*B vs.* the normalized surface density, gave $\kappa = -11.7 \pm 0.5$ with a substantially improved correlation coefficient, $r = 0.96$, suggesting that dehydration and other surface-density independent factors can be effectively accounted for by the "bulk phase" permeability model.

In the limit of random orientation of chain molecules in a lipid bilayer the order parameter, S_{mol}, approaches zero corresponding to a normalized surface density of 0.333, according to Dill's lattice model (*cf.*, Eq. 5). This state closely resembles that in a bulk hydrocarbon solvent. The extrapolated value of the permeability coefficient in this limiting case from the plot in Fig. 5*B* is 2.5×10^{-1} cm/sec. This value is significantly larger than the value predicted by the "bulk phase" model at the same reference temperature as presented in Fig. 3, $P_{bulk} = 4.1 \times 10^{-2}$ cm/sec. Apart from some unknown factors affecting this simple "bulk phase" estimate such as medium density, viscosity and experimental uncertainty, the discrepancy can also be rationalized by the difference of the chemical environment in the bilayer barrier from that of bulk decane. Previous studies reported by the authors demonstrated that the barrier domain within egg PC bilayers is more polar/polarizable than a fully saturated hydrocarbon possibly due to the relatively high concentration of double bonds in egg PC lipids and/or the close proximity of the barrier domain to the dipolar interface.

Thus, the barrier domain was shown to more closely resemble an unsaturated hydrocarbon solvent, 1,9-decadiene (Xiang & Anderson, 1994*b*; Xiang & Anderson, 1994*c*). In the present study, decane was initially chosen as a reference solvent because the alkyl chains within DMPC are fully saturated. However, cholesterol has a single double bond, and the extent to which the effective barrier polarity is influenced by its proximity to the interface remains unknown. Thus, decadiene may be a more appropriate bulk reference solvent even for DMPC:cholesterol bilayers. Indeed, the -OH group contribution in DMPC:cholesterol bilayers, determined recently in these laboratories, suggests that the DMPC:cholesterol barrier domain closely resembles 1,9-decadiene (Xiang & Anderson, 1995*a*). Acetic acid has a partition coefficient of $(1.3 \pm 0.2) \times 10^{-3}$ between decadiene and water, 4.2-fold larger than that between decane and water (Xiang & Anderson, 1994*b*). Using decadiene as a reference solvent and assuming that acetic acid has similar diffusion behavior in decadiene, a "bulk phase" permeability, P_{bulk}, of 2.1×10^{-1} cm/sec is estimated, in close agreement with the value extrapolated from Fig. 5*B*.

It is important to explore the molecular basis for the linearity in the relationship between the corrected permeability coefficient and surface density. In the Cohen-Turnbull diffusion theory (Cohen & Turnbull, 1959; Turnbull & Cohen, 1970) translational diffusion of a molecule occurs when statistical redistribution of free volume opens up a void of a critical size in the immediate vicinity of the molecule. The diffusion coefficient is thus proportional to the probability density of finding a free volume with a size equal to the size of the diffusing molecule V and can be formulated as

$$D = D_o e^{-\gamma V/V_f} \tag{8}$$

where D_o is a constant depending on the average kinetic velocity of the diffusant and γ is a numerical factor needed to correct for the overlap of free volume. V_f is the mean free volume in the medium. An expression which accounts for the free volume changes associated with changes in surface density has been derived by Cantor and Dill (1986) for phospholipid melting processes

$$v_f = V_1[1 - ae^{-k_s(\sigma-1)}] \tag{9}$$

where $V_1 = 1.23$ is a constant characterizing a reduced volume of amorphous alkane liquids and a is a function of V_1 and the depth in the bilayer interior. A linear fit of the log D data *vs.* surface density obtained in Eq. 8 using Eq. 9 in the surface density range of 0.5 to 0.9 gives a correlation coefficient of 0.997.

The barrier domain/water partition coefficient of permeant is also expected to vary with surface density, as determined by the changes in interaction energy between the solute and molecules composing the lipid bilayer with changes in bilayer order. This interaction energy can be divided into two parts, the attractive and the repulsive energy. The presence of a solute molecule in the bilayer would disrupt local chain order, as characterized by the overall order parameter of the chain molecule in the bilayer, $S = \Sigma_i (3/2 \cos^2\theta_i - 1/2)$, where θ_i is the angle between the normal to the bilayer surface and the normal to the plane spanned by two C_i-H bonds. According to the Marcelja mean-field model (Marcelja, 1974), the attractive energy (E_{disp}) in a lipid bilayer is related to S by

$$E_{\text{disp}} = -\Phi S \tag{10}$$

where Φ is a molecular field constant.

The lattice theory developed by Marqusee and Dill (1986) predicts that solute partitioning will also decrease with increasing surface density. More recently the authors have developed a statistical mechanical theory (Xiang & Anderson, 1994*a*) in which the more important repulsive interaction is described as a linear function of the lateral pressure $P_\perp$ in the bilayer membrane. Accordingly, the excluded-volume partition coefficient can be written as

$$K_{w\to m} = K_o e^{-2(p_\perp - 1)V/3k_BT} \tag{11}$$

where K_o is the partition coefficient at a lateral pressure of 1 bar (i.e., the partition coefficient between a bulk reference solvent and water at a constant pressure of 1 bar). Both experimental data and theoretical models (e.g., the lattice model) have shown that lateral pressure increases steeply with surface density (White & King, 1985; Cantor & Dill, 1986). The lateral pressure-surface density isotherm is nonlinear over a broad range of surface densities (0.16–1.0) with a slope which increases with surface density. However, in a more limited range of surface density as in the present study (0.58–0.88), a linear relationship may be a reasonable approximation (Marqusee & Dill, 1986).

Since the state of chain packing in lipid bilayers plays an important role in determining a solute's partition coefficient as well as its diffusivity, similar qualitative trends in solute partitioning with surface density would be expected. A plot of the logarithm of the membrane/water partition coefficient for acetic acid as a function of σ, shown in Fig. 6, confirms this expectation. A linear least-squares fit of the data in Fig. 6 gives a slope of $\kappa = -4.6 \pm 0.3$ with a correlation coefficient of 0.95. This slope is 2.7-fold smaller than the slope for the log permeability-surface density profile (-12.4 ± 1.1). Similar correlations have been observed between the partition coefficient for nonpolar solutes (e.g., hexane and benzene) and surface density in the same lipid bilayer systems (DeYoung & Dill, 1988; DeYoung & Dill, 1990), but again the changes in partition coefficient were modest. The amplified effects of surface density on per-

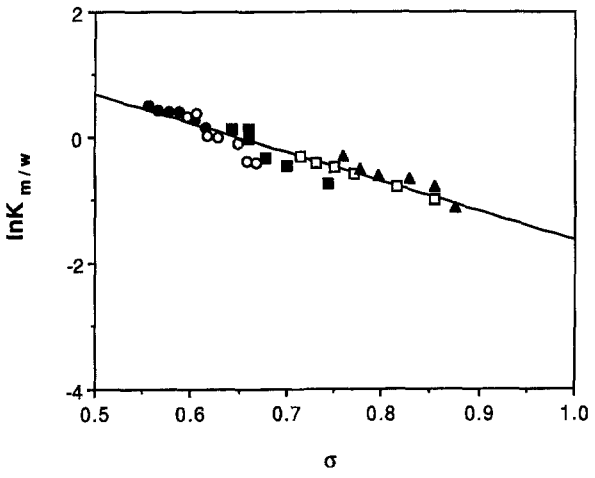

Fig. 6. Partition coefficient for acetic acid between a DMPC:cholesterol lipid bilayer and water *vs.* surface density σ: ●: $X_{chol} = 0.00$; ○: $X_{chol} = 0.10$; ■: $X_{chol} = 0.20$; □: $X_{chol} = 0.30$; and ▲: $X_{chol} = 0.40$.

meability coefficients relative to partition coefficients can be rationalized by arguments similar to those used to account for differences in sensitivity to temperature: (i) variation in surface density may have an additional effect on solute diffusivity in lipid bilayers which does not affect solute partitioning; and (ii) partition coefficients reflect the properties of different (i.e., less highly ordered) regions within the bilayer than permeability coefficients. Nonpolar solutes partition preferentially into the center of the lipid bilayer where the acyl chains in the lipid bilayers are relatively disordered (McIntosh et al., 1980; White et al., 1981; Pope et al., 1984; Marqusee & Dill, 1986; Xiang & Anderson, 1994*a*). Acetic acid is likely to localize preferentially at the bilayer/water interface with only partial penetration into the highly ordered barrier region (Miller et al., 1977; Pope et al., 1984). Nevertheless, the present results reveal that even for a polar, hydrogen-bonding solute, surface density is a dominant factor governing partitioning into lipid bilayers varying in cholesterol composition and temperature.

In summary, this work represents an important step in efforts to understand the molecular mechanisms of solute partitioning into and permeation across lipid bilayer membranes. The quantitative relationships established between solute partition and permeability coefficients and phospholipid bilayer surface density support the hypothesis that chain ordering in lipid bilayers (or surface density) is a major determinant for *both* molecular uptake and transport across lipid bilayers. The dehydration process is a minor component to overall permeability. These conclusions appear to be independent of the nature of the thermodynamic variables used to alter surface density (e.g., cholesterol concentration and temperature).

This work was supported by grants from Glaxo, INTERx/Merck, and University of Utah Research Committee.

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