BRIEF COMMUNICATION

THE NONEFFECT OF A LARGE LINEAR HYDROCARBON, SQUALENE, ON THE PHOSPHATIDYLCHOLINE PACKING STRUCTURE

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ABSTRACT The interaction of squalene with liposomes and monolayers of dipalmitoyl phosphatidylcholine (DPL) has been studied by differential scanning calorimetry, Raman spectroscopy, and surface potential measurements. Mole ratios of squalene to DPL up to 9 to 1 were studied. In contrast to small, nonpolar molecules, which profoundly influence the structure of lipid bilayers as detected by changes in both their thermodynamic phase transition parameters and membrane fluidity, this large, nonpolar, linear hydrocarbon is devoid of such influences. It is clear from our data that a large nonpolar molecule such as squalene, having no polar group that might anchor it to the aqueous interface, cannot intercalate between the acyl chains either below or above the phase transition of DPL. This behavior is not compatible with models that treat the bilayer interior as a bulk hydrocarbon, and suggests that great caution should be exercised in extrapolating partition coefficients based on bulk hydrocarbon measurements to lipid bilayers.

INTRODUCTION

Since the permeabilities of biological membranes to many molecules are proportional to the oil/water partition coefficient of such molecules, it has generally been assumed that the interior of these membranes resembles an organic liquid (1–3). More recently it has been accepted that this behavior follows from the fact that a substantial proportion of most biological membranes are in the form of lipid bilayers. It is understandable, therefore, that descriptions of most lipid bilayers (liposomes, planar lipid bilayers) likewise assume that its interior resembles some isotropic organic liquid, such as olive oil, ether, octanol, or hexadecane (2–5).

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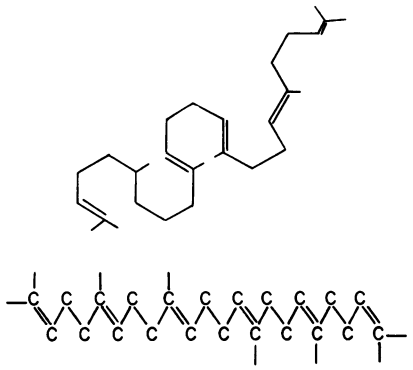

FIGURE 1 A picture of the molecule squalene. Squalene ($C_{30}H_{50}$) is a liquid at room temperature. It has a density of 0.8584 g/cm^3 and an extended length of about 29 Å. Unlike cholesterol, which has about the same molecular volume, squalene lacks a hydroxyl group, which may anchor it to the interface. Note that the rings are not closed.

The lipid bilayer, in contrast to a bulk organic liquid, is inherently anisotropic and more ordered than isotropic liquids. These facts may not be important in ascertaining solvent and permeability properties of molecules small in size with respect to the acyl chains of the lipid molecules; there the bulk liquid assumption may well be justified (5, 6). This assumption clearly becomes questionable when the size of the molecule is comparable to the size of an acyl chain of a lipid molecule. The dissolution of such molecules into bilayers may not be favored because of the severe restriction of their motion when placed into a relatively "ordered liquid" such as a lipid bilayer. Indeed, evidence has been gathered that this may occur in lipid bilayers for the *n*-alkanes (5, 7, 8).

It has been documented that, in the case of lipid bilayers that exhibit well-defined "gel-" liquid crystal phase transitions, equilibration with many small nonpolar molecules leads to increases in membrane fluidity and decreases in transition temperature (i.e., general increase of disorder in the system) (9–10).

We have chosen squalene (Fig. 1), a large, linear, nonpolar molecule, to test whether it dissolves in the bilayer like small hydrophobic molecules.

We have examined the interaction of squalene with DPL by three techniques, the first being differential scanning calorimetry (DSC). Both the transition temperature, T_m, and enthalpy, ΔH_m, of the main endothermic transition can be monitored by these techniques. Changes in these parameters are useful in determining how the bilayer is affected by the presence of foreign molecules (11). The second technique is a measurement of the total surface potential of a spread monolayer of dipalmitoyl phosphatidylcholine (DPL) as a function of temperature at the air-water interface. In addition to measuring T_m, this technique measures the area change accompanying the transition (12). The transition temperature obtained by this technique is sensitive to the spreading solvent (12). The third technique is Raman spectroscopy. The DPL Raman spectrum in the region around 1,100 cm^{-1} contains bands due to all-*trans* and random C-C stretch. These bands have been shown to be sensitive to the "fluidity" of the lipid

hydrocarbon chains (13, 14). In particular we have examined the change in the all-*trans* to random C-C stretch peak height intensity ratios $I_{1,064}/I_{1,089}$ and $I_{1,128}/I_{1,089}$ as a direct measure of the fluidity of the nonpolar phase of DPL bilayers containing various squalene concentrations.

We conclude that squalene at the mole ratios examined is immiscible with the acyl chains of DPL bilayers or monolayers.

METHODS

1,2-L-α-dipalmitoyl phosphatidylcholine (DPL) was purchased from Supelco, Inc., (Bellefonte, Penn.) and used without further purification. This material gave a single spot of thin-layer chromatography. Squalene was purchased from Sigma Chemical Co., (St. Louis, Mo.) and passed through an alumina column before use to remove polar impurities. Water was twice distilled, the second time in an all-glass apparatus.

Calorimetric measurements were performed with a Perkin-Elmer DSC-1B (Perkin-Elmer Co., Instrument Div., Norwalk, Conn.). Samples used for this part of the study were prepared by mixing the desired amounts of lipid, squalene, and water in a volatile sample holder. The sample was then heated to 60°C for 2 h to facilitate mixing, and air-cooled. Other samples were prepared by dissolving DPL and squalene in chloroform, evaporating off the chloroform, and then proceeding as above. Details of the sampling procedure have been described elsewhere (12).

The Raman spectrometer has been described elsewhere (15). Generally, the 488.0 and 514.5-nm lines of an argon ion laser were used. The laser was generally run at a power of 600 nW with nm lines of an argon ion laser were used. The laser was generally run at a power of 600 nW with the monochrometer slits at 200 μm. The samples were analyzed at 24°C and 50°C. The maximum difference in peak height intensity ratios measured from the spectral base line during independent runs was ± 0.1. Samples were prepared by mixing the desired amounts of lipid and squalene in chloroform, evaporating off the chloroform, reweighing the sample, and then adding water. The lipid and solvent-to-water ratio in all cases was 1:4. The samples were then equilibrated at 60°C for 2 h.

The surface potential vs. temperature experiments are described in detail elsewhere (12). The surface potential was measured by a polonium air electrode and a Ag/AgCl electrode in the aqueous phase. The temperature was measured by a thermistor probe located near the surface of the monolayer with an accuracy of ± 0.2°C. The aqueous phase was stirred. DPL-squalene at the tested mole ratios were dissolved in chloroform-methanol 2/1 (vol/vol) and deposited in excess along the side of the trough. The samples were then cycled through their transition temperature.

RESULTS

Differential Scanning Calorimetry

Table I shows the thermodynamic data for DPL-water (excess) alone and in the presence of squalene at mole ratios (squalene:DPL) of 1:2.0, 1:3.0, and 1:4.0. The thermodynamic transition parameters for DPL-water (excess) generally agree with the values found by Jacobson and Papahadjopoulos (16) but are about 0.7 kcal/mol lower than other reported values (17). At the squalene concentrations investigated, transition parameters are not significantly altered. The sharpness of the DPL peak at all mole fractions, ($W_{1/2H}$) also indicates little interaction of squalene with the phosphatidylcholine hydrocarbon region (11). Similar results were obtained on heating and cooling cycles.

TABLE I

THERMODYNAMIC DATA FOR DPL-WATER (EXCESS)—SQUALENE

Squalene/DPL	T_m	ΔH_m	ΔS_m	$W_{1/2H}$
mol/mol	*°C*	*kcal/mol*	*eμ*	*°C*
0	42.1	7.5	23.9	0.6
1:4.0	42.5	7.1	22.4	0.8
1:3.0	42.0	7.1	22.5	0.7
1:2.0	41.5	7.2	23.0	0.8

Raman Spectroscopy

At 24°C and 50°C, squalene and DPL in mole ratios (squalene:DPL) of 1:9, 1:4, 3:7, 2:3, and 1:1 were analyzed. The presence of squalene did not cause the DPL Raman peak height intensity ratios $I_{1,064}/I_{1,089}$ and $I_{1,128}/I_{1,089}$ to change appreciably from those of DPL alone in water (2.0 and 1.6, respectively at 24°C, and 0.9 and 0.4 at 50°C) (Fig. 2). This is in marked contrast to the effect of cholesterol on the Raman spectrum of phosphatidylcholine (13), where even at room temperature the two peak height intensity ratios $I_{1,064}/I_{1,089}$ and $I_{1,128}/I_{1,089}$ were reduced, indicating a more disordered phosphatidylcholine hydrocarbon chain structure. Fig. 3 shows the Raman spectra of DPL in water alone and in a 1:1 mole ratio with squalene at 24°C. The peak height intensity ratios in the two cases differed by ±0.1 or less. The squalene Raman spectrum (also shown in Fig. 1) did not cause an appreciable interference in

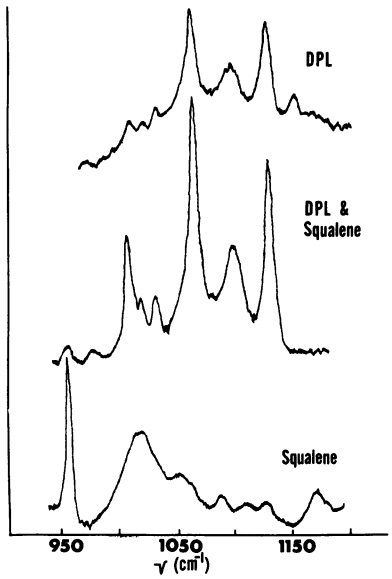

FIGURE 2 Raman spectra of DPL in H_2O and in a 1:1 mole ratio with squalene in H_2O. Raman spectrum of pure squalene at three times the intensity of the pure DPL and DPL + squalene spectra are included. All spectra were taken at 24°C.

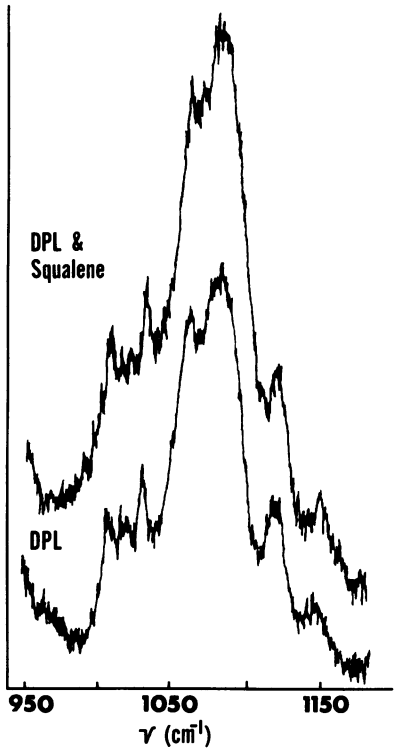

FIGURE 3 Raman spectra of DPL in H_2O and in a 4:1 mole ratio with squalene in H_2O. Spectra were recorded with the samples at about 50°C.

the DPL Raman spectrum. Features in the spectral region, 1,000–1,050 for the DPL-squalene mixtures are probably grating ghosts.

Surface Potential

Monolayers of DPL-squalene were spread in excess, at mole ratios 1:1, 1:2, and 1:4, with the spreading solvent being chloroform:methanol 2/1 (vol/vol). The monolayers were usually spread at 25°C, although some were spread at 45°C with identical results. Once the surface potential reached a steady value, the temperature was increased or decreased. Fig. 4 shows a surface potential vs. temperature heating and cooling cycle for a DPL:squalene 1:1 mole ratio. In this figure the surface potential decreases gradually from 25°C until 40°C, where it abruptly drops from 530 to 490 mV. The cooling curve follows the heating curve. These curves are similar to the ones found for DPL alone (12).

DISCUSSION

The results show that squalene and DPL-water (excess) form two immiscible phases at the mole fractions examined, despite the large oil-water partition coefficient presumed for squalene (18). In this regard caution must be used in extrapolating mem-

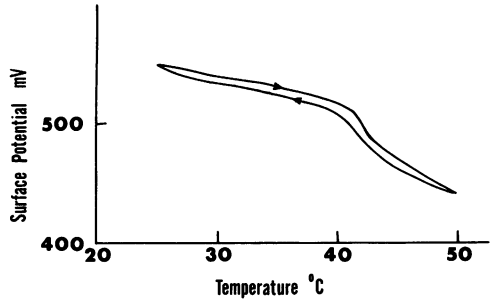

FIGURE 4 Surface potential vs. temperature curve of a DPL-squalene (1:1) monolayer. The monolayer was spread at 24°C in excess. The spreading solvent was chloroform-methanol 2:1 (vol/vol).

brane water partition coefficients from organic liquid-water partition coefficients, especially for large nonpolar molecules.

Our results also show that squalene is excluded from positions between (axial) the acyl chains of DPL both above and below the phase transition. The geometry of the squalene-DPL (water) phase could conceivably be represented either by a thick layer of squalene between the two monolayers or by a system in which the bulk of the DPL remains pristine and most of the squalene is in a few droplets covered by a negligible fraction of the DPL. The former configuration appears much less likely then the latter, since it would require that a bilayer adhering to a layer of squalene between adjacent monolayers be identical to an undoped bilayer. Because the attractive interaction between acyl chains are diminished at oil-water interfaces (19), we think it unlikely that the hydrocarbon is located in this region. Furthermore, the free energy of transfer of squalene from itself into the bilayer should not be energetically favored, principally because of the large entropy decrease that would accompany this transfer. This entropy decrease has been measured in planar lipid bilayers for molecules as small as *n*-octane and as large as *n*-octadecane (7, 20) and in phospholipid vesicles for a molecule as small as *n*-hexane (8). Another factor militating against solution of large nonpolar molecules into the hydrophobic region of a membrane (with a fixed number of molecules) is that it will necessarily expose some acyl chain hydrocarbon to water, consequently increasing the energy of the membrane interface. Thus it seems probable that essentially all of the squalene in the systems that we have studied lies in a few monolayer-covered droplets and the amount of DPL in this state is a small proportion of the total.

The question of whether any squalene resides in regions of the bilayers positioned axially to the acyl chains in DPL can definitively be answered in the negative. Neither the phase transition parameters nor changes in *trans*-gauche configurations of the fatty acyl chains were affected by squalene. While this may not be surprising for DPL below the phase transition, since even small molecules such as TEMPO are excluded from the bilayer in the gel phase (21), it is somewhat surprising that squalene is excluded from the liquid crystalline phase. This was demonstrated by DSC measure-

ment of the phase transition temperature from either above or below T_m. If squalene intercalated between chains at temperatures above T_m, cooling would reveal modified transition behavior due to the extrusion of the squalene. In addition, the Raman spectra of squalene above T_m yields direct evidence for the absence of squalene from DPL phase.

White has shown that the length, rather than the volume, of a hydrocarbon is the important parameter that correlates with solubility in planar lipid bilayers (22). He has intimated that a linear chain of 21 carbons should be totally immiscible in a bilayer. Squalene has 30 carbons and an extended length of 29 Å (23), so in this context the results are not surprising.

These data also emphasize the critical role that a single polar functional group can play. Cholesterol is about the same size as squalene; yet this molecule, with one hydroxyl group, is readily accommodated into lipid bilayers and monolayers at much lower mole fractions than we used in our squalene experiments. Cholesterol exhibits dramatic effects on the phase transition of DPL, as determined by DSC as well as by the Raman spectra (13, 24). Squalene also lacks a freezing point depression, as seen with the small chain length alcohols such as butanol, and the elevation of the transition temperature, as seen with the longer chain alcohols such as *n*-decanol (5).

For the reasons just stated we suggest that conclusions obtained from fluorescence measurements using large nonpolar bulky molecules such as pyrene should be reconsidered with the caveat that the probe may be reporting on a region of the bilayer not representative of the whole bilayer (25, 26).

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REFERENCES

1. DIAMOND, J. M., and E. M. WRIGHT. 1969. Biological membranes. The physical basis of ion and nonelectrolyte selectivity. *Annu. Rev. Physiol.* **31**:581–646.
2. COHEN, B. 1975. The permeability of liposomes to non-electrolytes. I. Activation energies for permeation. *J. Membr. Biol.* **20**:205–234.
3. FINKELSTEIN, A. 1976. Water and nonelectrolyte permeability of lipid bilayer membranes. *J. Gen. Physiol.* **68**:127–135.
4. WOLOSIN, J. M., and H. GINSBURG. 1975. The permeation of organic acids through lecithin bilayers. Resemblence to diffusion in polymers. *Biochim. Biophys. Acta.* **389**:20–33.
5. LEE, A. C. 1976. Interations between anesthetics and lipid mixtures. Normal alcohols. *Biochemistry.* **15**:2448–2454.
6. KATZ, Y. 1976. Solvent properties and membrane interactions in phospholipid membranes. *Biophys. J.* **16**:52*a*. (Abstr.).
7. WHITE, S. H. 1976. The lipid bilayer as a "solvent" for small hydrophobic molecules. *Nature (Lond.).* **262**:421–422.
8. SIMON, S. A., W. H. STONE, and P. BUSTO-LATORRE. 1977. A thermodynamic study of the partition of *n*-hexane into lecithin and lecithin-cholesterol bilayers. *Biochim. Biophys. Acta.* In press.

9. TRUDELL, J. R., W. L. HUBBELL, and E. N. COHEN. 1973. The effect of two inhalation anesthetics on the order of spin-labeled phospholipid vesicles. *Biochim. Biophys. Acta.* **291**:321–327.
10. JAIN, M. K., N. WU, and L. V. WRAY. 1975. Drug induced changes in bilayers as a possible mode of action of membrane expanding drugs. *Nature (Lond.).* **255**:494–496.
11. PAPAHADJOPOULOS, D., M. MUSCARELLO, E. H. EYLAR, and T. ISAC. 1975. Effect of proteins on the thermotropic phase transitions of phospholipid membranes. *Biochim. Biophys. Acta.* **401**:417–435.
12. SIMON, S. A., L. J. LIS, J. W. KAUFFMAN, and R. C. MACDONALD. 1975. A calorimetric and monolayer investigation of ions on the thermodynamic properties of phosphatidylcholine. *Biochim. Biophys. Acta.* **375**:317–326.
13. LIPPET, J. L., and W. L. PETICOLAS. 1971. Laser Raman investigation of the effect of cholesterol on conformational changes in dipalmitoyl lecithin multilayers. *Proc. Natl. Acad. Sci. U.S.A.* **68**: 1572–1576.
14. BROWN, K. G., W. L. PETICOLAS, and E. BROWN. 1973. Raman studies of conformational changes in model membrane systems. *Biochem. Biophys. Res. Commun.* **54**:358–364.
15. LIS, L. J., J. W. KAUFFMAN, and D. F. SHRIVER. 1975. Effect of ions on phospholipid layer structure as indicated by Raman spectroscopy. *Biochim. Biophys. Acta.* **406**:453–464.
16. JACOBSON, K., and D. PAPAHADJOPOULOS. 1975. Phase transitions and phase separations in phospholipid membranes induced by changes in temperature, pH, and concentration of bivalent ion. *Biochemistry.* **14**:152–173.
17. CHAPMAN, D., R. M. WILLIAMS, and B. D. LABBROOKE. 1967. Physical studies of phospholipids. VI. Thermotropic and lyotropic polymorphism of some 1,2-diacylphosphatidylcholine, (lecithin). *Chem. Phys. Lipids.* **1**:445–475.
18. REYNOLDS, J. H., D. B. GILBERT, and C. T. TANFORD. 1974. Empirical correlation between hydrophobic free energy and aqueous cavity surface area. *Proc. Natl. Acad. Sci. U.S.A.* **71**:2925–2928.
19. DAVIES, J. T., and E. K. RIDEAL. 1961. Interfacial Phenomena. Academic Press, Inc., New York. 227–240.
20. WHITE, S. H. 1977. Solubility of *n*-alkanes in planar bilayer membranes. *Biophys. J.*, **17**:129*a*. (Abstr.).
21. SHIMSHICK, E. J., and H. M. MCCONNELL. 1973. Lateral phase separation in phospholipid membranes. *Biochemistry.* **12**:2351–2360.
22. WHITE, S. H. 1977. Studies of the physical chemistry of planar lipid membranes using high precision measurements of specific capacitance. *Ann. N. Y. Acad. Sci.* In press.
23. LANJI, J. K., W. Z. PLACHY, and M. KATES. 1974. Lipid interactions in membranes of extremely halophilic bacteria. II. Modification of the bilayer structure by squalene. *Biochemistry.* **13**:4914–4920.
24. PAPAHADJAPOULOS, D., and H. K. KIMELBERG. 1973. Phospholipid vesicles (liposomes) as models for biological membranes. Their properties and interactions with cholesterol and proteins. *Rec. Prog. Surf. Sci.* **4**:141–232.
25. VANDERKOOI, J. M., and J. B. CALLIS. 1974. Pyrene: a probe of lateral diffusion in the hydrophobic region of a membrane. *Biochemistry.* **13**:4000–4006.
26. SHINITZKY, M., and M. INBAR. 1976. Microviscosity parameters and protein mobility in biological membranes. *Biochim. Biophys. Acta.* **433**:133–149.