A ring and the C1 of the sugar.

Conclusions

We have established that Yb(III) binds to a unique position on the C_{11}, C_{12} oxygens in the plane of the ring system of Adm. The possibility that the Yb(III) ion binds to the other quinone, semiquinone system (C_5, C_6 oxygens) can be excluded on the basis of the distances determined from the T_1 data, i.e., there is only one reasonable fit to the T_1 data. This is the model in which the Yb(III) resides at the C_{11}, C_{12} binding site in Adm. The results

of this study illustrate the potential utility of analyzing Yb-(III)-induced relaxation rate enhancements in terms of internuclear distances. We are currently extending the analysis of this paper to determine the conformation of the sugar ring of Adm in methanol.

Behavior of Hexane Dissolved in Dioleoylphosphatidylcholine Bilayers: An NMR and Calorimetric Study[†]

Russell E. Jacobs* and Stephen H. White

Contribution from the Department of Physiology and Biophysics, University of California, Irvine, California 92717. Received April 18, 1984

Abstract: Deuterium nuclear magnetic resonance (NMR) spectroscopy and differential scanning calorimetry (DSC) have been used to examine the behavior of 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) bilayers to which hexane has been added. This work represents an extension of an earlier study of hexane dissolved in dimyristoylphosphatidylcholine (DMPC) bilayers. Both differences and similarities in the two bilayers are oberved. DSC thermograms indicate that hexane incorporated into DOPC bilayers behaves as a simple impurity—the lipid phase transition is broadened and shifted to lower temperatures by the addition of hexane. Deuterium NMR spectra of perdeuterated hexane in the bilayers show three overlapping powder patterns, indicating that on average both ends of the molecule are experiencing the same environment. The temperature dependence of the hexane deuterium quadrupole splittings exhibits a maximum at the lipid bilayer phase transition temperature. The hexane powder patterns coalesce into a single line under conditions of low temperature and high concentration, indicating the onset of isotropic motion. Deuterium NMR spectra of 1,2-(9′,10′-$^2H_2$)DOPC consist of four overlapping powder patterns which change little upon the addition of up to 50 mol % hexane. Thus, the bilayer stays intact and the motional characteristics of the DOPC double bonds are not changed by the presence of even an equimolar mixture of the alkane.

The lipid bilayer constitutes the principal structural features of the biological membrane.[1] A variety of hydrophobic components exist naturally within the bilayer (e.g., membrane proteins and cholesterol)[2,3] or can be introduced artificially (e.g., general anesthetics).[4,5] The lipid bilayer is essentially a "solvent" for hydrophobic "solute" molecules. A quantitative understanding of the solvent properties of lipid bilayers is essential for understanding membrane structure, the insertion and translocation of proteins into and across membranes, and the mechanisms of membrane active drugs. In order to characterize the interactions between bilayer lipid and hydrophobic components in detail, we are examining simple model systems. In the work reported here, hexane is employed as a prototypal hydrophobic molecule and the bilayer is constructed from a single type of lipid. In a previous study, we examined the hexane/DMPC bilayer system.[6] We found that the alkane behaves in a very nonideal manner and that an unusually large amount of hexane could be incorporated into the bilayer without affecting the motional behavior of the lipids.

We present here information about the behavior of the hexane/DOPC bilayer system. Differential scanning calorimetry thermograms indicate that hexane acts as a simple impurity—even at concentrations in the bilayer equimolar with the lipid. ^{2}H NMR powder patterns give direct information about the average order imposed upon the alkane by the bilayer and, by inference, information about the types of anisotropic motion being executed by the labeled molecule.[7,8] The ^{2}H NMR spectra of bilayers of DOPC labeled at the double bonds are essentially unaffected by the addition of hexane. The spectra consist of four overlapping powder patterns, one from each of the four deuterons. ^{2}H NMR

spectra of perdeuterated hexane dissolved in DOPC bilayers reveal that the types of motion that this small molecule undergoes in the bilayer are strong functions of both temperature and concentration.

Experimental Procedure

^{2}H labeled and unlabeled DOPC were purchased from Avanti Polar-Lipids, Inc. (Birmingham, AL), deuterium-depleted water (^{2}H content = 1% of natural abundance) from Sigma (St. Louis, MO), hexane (pesticide grade) from Burdick and Jackson Laboratories, Inc. (Muskegon, MI), perdeuterated hexane from KOR Isotopes, Inc. (Cambridge, MA), and ^{14}C-labeled DOPC was from New England Nuclear (Cambridge, MA). Tritiated hexane was synthesized from 1-bromohexane via reduction with LiAl^{3}H$_4$ and LiAl^{2}H$_4$. All alkanes used were checked for purity by using gas chromatography and found to be greater than 99% pure. Thin-layer chromatography was used to monitor lipid purity.

A series of mixtures of DOPC, hexane, and ^{2}H-depleted water were prepared. Samples were prepared in essentially the same manner as

---

(1) Singer, S. J.; Nicolson, G. L. *Science (Washington, D.C.)* **1972**, *175*, 720–731.
(2) Silvius, J. R. In "Lipid-Peptide Interactions"; Jost, P. C., Griffiths, O. H., Eds.; Wiley: New York, 1982; Vol. 2, Chapter 7.
(3) Lentz, B. R.; Barrow, D. A.; Hoechli, M. *Biochemistry* **1980**, *19*, 1943–1954.
(4) Hill, M. W. "Molecular Mechanisms in General Anesthesia"; Halsey, M. J., Miller, R. A., Sutton, J. A., Eds.; Churchill-Livingstone: Edinburg, England, 1974.
(5) Mullins, L. J. "Molecular Mechanisms of Anesthesia"; Fink, B. R. Ed.; Raven Press: New York, 1975; Vol. 1, p 237.
(6) Jacobs, R. E.; White, S. H. *J. Am. Chem. Soc.* **1984**, *106*, 915–920.
(7) Jacobs, R.; Oldfield, *Prog. Nucl. Magn. Reson. Spectrosc.* **1980**, *14*, 113–136.
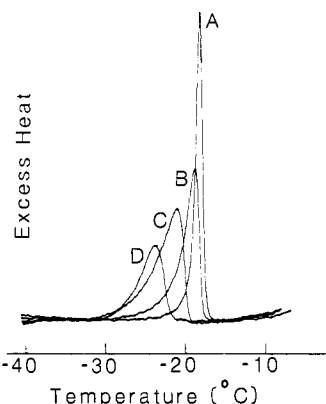(8) Seelig, J. *Q. Rev. Biophys.* **1977**, *10*, 353.

Figure 1. DSC thermograms of multilamellar liposomes of DOPC containing various concentrations of hexane: A, no hexane; B, 3 mol %; C, 27 mol %; D, 45 mol %. The enthalpies in kilocalories per mole of DOPC for the endotherms shown: A, 8.5; B, 8.2; C, 8.0; D, 7.6.

described previously.[6] Briefly, hexane (at various partial pressures) and ^{2}H-depleted water were allowed to equilibrate with the lipid from 12 h. The lipid was then taken up with about 300 μL for ^{2}H-depleted water and sealed in the NMR tube. In all cases the DOPC was doped with a small amount of (^{14}C)DOPC and the alkane with (^{3}H)hexane, and the relative amounts of the two components were determined by radioactive counting methods. The mole percent hexane was then calculated as the moles of hexane divided by the total number of moles of lipid plus hexane. The uncertainty in the mole percent hexane noted below is 2 mol %. When pure hexane was used in the equilibrium (partial pressure of hexane equal to unity), 50 mol % hexane was incorporated into the DOPC bilayer.

^{2}H NMR spectra were obtained at the NSF Southern California Regional NMR Center on a modified Bruker WM-500 spectrometer in the Fourier transform mode at 76.8 MHz (magnetic field strength of 11.7 T). All ^{2}H spectra were taken by using the quadrupole echo technique[9,10] with a t_{echo} = 40 μs and a 90° pulse of 6–9 μs. The pulse repetition rate was one per second. The number of scans per spectrum varied from 1000 to 10 000. All free induction decays were processed with an exponential multiplication factor of 40 Hz. The data acquisition rate was 166 666 Hz. Sample temeperature was controlled with a flow of temperature-regulated nitrogen gas. In all cases the sample was allowed to reach thermal equilibrium before data acquisition was begun. Typically, this took 20 min after the probe temperature had stabilized at the desired temperature. The relative intensities of the overlapping powder patterns observed were obtained via a spectral simulation routine.[11]

The differential scanning calorimetry was performed on a Perkin-Elmer DSC-2B with a liquid nitrogen cooling accessory. A typical DSC sample contained about 2 mg of DOPC/hexane and 5 mg of water. For each thermogram the sample was cooled to −43 °C, held there for 15–30 min, and then heated to 30 °C at 1.25 °C/min. In Figure 1 the endotherm due to water freezing has been omitted. Low-temperature thermograms were run in essentially the same manner except that the temperature span was from −113 to −43 °C.

Results

The addition of hexane to DOPC bilayers causes the lipid phase transition endotherm to shift to lower temperatures and broaden as shown in Figure 1. The enthalpy of the transition is approximately 8 kcal/mol DOPC and is decreased slightly by the addition of the alkane (see Figure 1). No heat absorption, other than that shown and an ice–liquid water endotherm, was observed between −113 and 30 °C.

Figures 2 and 3 show ^{2}H NMR spectra of perdeuterated hexane dissolved in DOPC multilayers over a range of concentrations and temperatures. At relatively low concentrations (less than 30 mol %) and high temperatures (greater than −18 °C, the phase transition temperature, T_m, for pure DOPC bilayers), the spectra are composed of three overlapping powder patterns in an intensity ratio of 3:2:2. The powder pattern with the smallest quadrupole
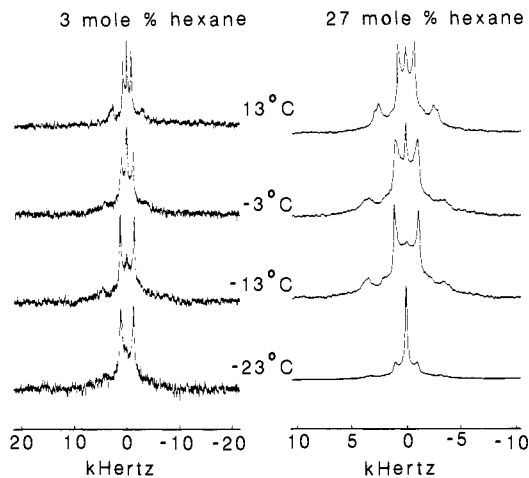
(9) Solomon, I. *Phys. Rev.* **1958**, *110*, 61–65.
(10) Davis, J. H.; Jeffery, K. R.; Bloom, M.; Valic, M. I.; Higgs, T. P. *Chem. Phys. Lett.* **1976**, *42*, 390–394.
(11) Kang, S. Y.; Gutowsky, H. S.; Hsung, J. C.; Jacobs, R.; King, T. S.; Rice, D.; Oldfield, E. *Biochemistry* **1979**, *18*, 3257.

Figure 2. ^{2}H NMR spectra of perdeuterated hexane in DOPC liposomes at several temperatures and two concentrations. The slightly off-center peak in the 3 mol % spectra comes from residual HOD.[6]
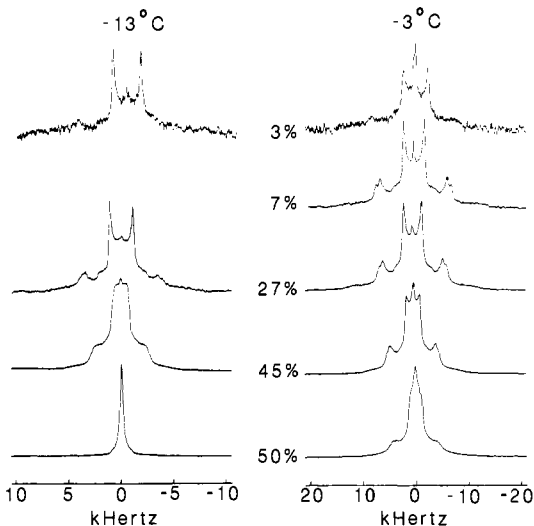

Figure 3. ^{2}H NMR spectra of perdeuterated hexane in DOPC liposomes at several concentrations and two temperatures.
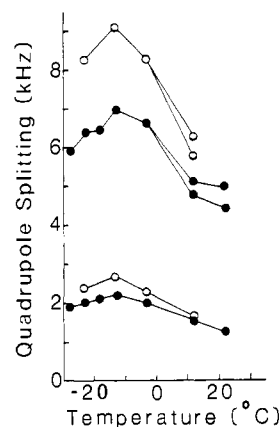

Figure 4. Plots of the innermost (lower traces) and outermost (upper traces) quadrupole splittings of perdeuterated hexane in DOPC liposomes as a function of temperature at two hexane concentrations: open circles, 5 mol %; closed circles, 20 mol %. The two outer powder patterns coalese below 0 °C, yielding a single quadrupole splitting.

splitting ($\Delta\nu_q$, the separation between peak maxima) is the most intense. In these figures each CD_2 and CD_3 group contributes a powder pattern doublet to the spectra. The order parameter S_{CD} is related to $\Delta\nu_q$ by

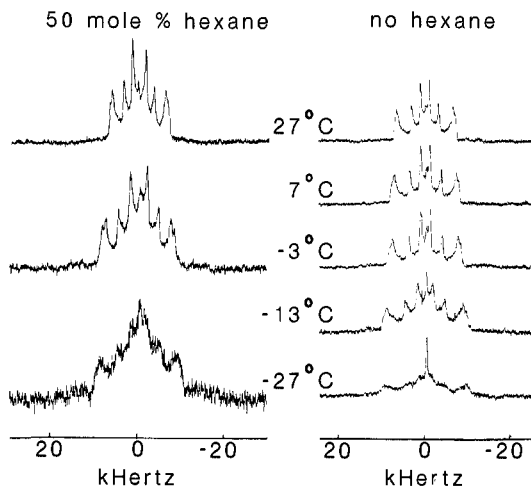$$\Delta\nu_q = \frac{3}{4}(e^2qQ/h)S_{CD}$$

Figure 5. ^{2}H NMR spectra of liposomes of pure 1,2-(9',10'-^{2}H$_2$)DOPC and after the incorporation of 50 mol % hexane.
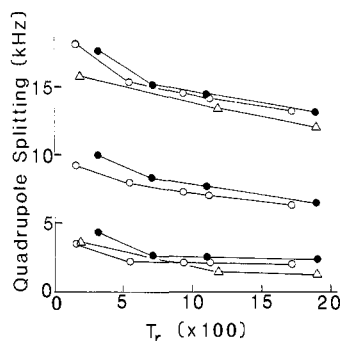

Figure 6. Plots of quadrupole splittings from liposomes of the double bond deuterated lipids as a function of reduced temperature: triangles, POPC;[16] closed circles, DOPC; open circles, DOPC plus 50 mol % hexane.

where e^2qQ/h is the static quadrupole coupling constant: 167 kHz for the C–^{2}H bond[12,13] or 175 kHz for an olefinic C–^{2}H bond.[14] At high hexane concentrations and low temperatures a central isotropic peak emerges as the dominant spectral feature. Plots of $\Delta\nu_q$ as a function of temperature (Figure 4) exhibit maxima at approximately the T_m of DOPC. Figure 4 also shows that an increase in hexane content causes a decrease in $\Delta\nu_q$ for the alkane.

^{2}H NMR spectra of 1,2-(9',10'-^{2}H$_2$)DOPC multilayers with 0 and 50 mol % hexane are shown in Figure 5. Four overlapping powder patterns of equal intensity are observed, although the outer two patterns have almost the same $\Delta\nu_q$. Seeling the co-workers[15,16] have measured ^{2}H NMR spectra for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) bilayers in which the oleic acyl chain has been deuterated at the double bond. They observe two powder patterns: one from each of the two olefinic deuterons. Figure 6 shows plots of $\Delta\nu_q$ for double-bond deuterated POPC and DOPC bilayers as a function of reduced temperature ($T_r = (T - T_m)/T_m$). We note that the inner most and outer most quadrupole splittings are substantially the same in the three systems shown: POPC, DOPC, and DOPC/hexane (50 mol % hexane).

Discussion

DMPC and DOPC possess the same head group (phosphatidylcholine) but different acyl chains: the former having two saturated chains 14 carbons long, while the latter has acyl chains 18 carbons long with a double bond halfway between the head

group and the chain end. This difference is reflected in the different physical properties of the bilayers the lipids form; e.g., their bilayer phase transition temperatures differ by 40 °C. A comparison of the consequences of adding a small hydrophobic molecule to these bilayer systems reveals interesting similarities and differences. In an earlier study[6] we found that DMPC bilayers would incorporate on the order of 30 mol % hexane before the bilayer structure collapsed. Because no significant change is seen in the ^{2}H NMR spectra of ^{2}H-labeled DOPC bilayers upon the incorporation of up to an equimolar mixture of hexane, the DOPC bilayer structure (as reported by the labeled double bonds) must be relatively unperturbed by the addition of this much alkane. At low hexane concentrations DSC thermograms indicate that the DMPC bilayer undergoes a phase separation at about 0 °C. Whereas, in the DOPC bilayers, hexane behaves as an impurity causing the bilayer phase transition endotherm to broaden and shift to lower temperatures. This behavior extends to equimolar amounts of incorporated hexane—the highest concentration we could obtain through our experimental protocol. Thus, both the DSC and NMR results imply that, compared to the DMPC bilayer, the DOPC bilayer is a much more accomodating structure: significantly more alkane can be incorporated with significantly less disruption.

Except for the lowest hexane concentrations, the temperature and concentration dependence of the ^{2}H NMR spectra of perdeuterated hexane incorporated into DOPC and DMPC bilayers follows the same general course. At low concentrations of hexane in DOPC bilayers (less than 7 mol %) relatively sharp ^{2}H NMR powder patterns are observed for the labeled hexane at all temperatures examined, even well into the gel phase of the DOPC bilayer. In the gel phase of the hexane/DMPC bilayer system at low hexane content almost all of the intensity in the spectrum of the deuterated hexane was in a central isotropic peak. This behavior is observed at higher hexane concentrations in the DOPC bilayer where an isotropic peak appears superimposed on the powder patterns as the sample temperature is lowered through its phase transition temperature (see Figure 2). Figure 3 reveals that at constant temperature the amount of isotropic hexane (as compared to the anisotropic spectral component) increases with the total concentration of hexane. In both bilayers at very high hexane contents there is an appreciable isotropic component even in the liquid crystalline phase of the bilayer. In DMPC bilayers the appearance of an isotropic peak in the ^{2}H NMR spectra appears to coincide with the phase separation within the bilayer as observed by DSC. This is not the case in the hexane/DOPC bilayer (no phase separation is observed in the DSC thermograms of this mixture), although in both systems the isotropic behavior is prevalent at low temperatures and high concentrations of the alkane. Recent neutron diffraction studies of oriented multilayers of liquid crystalline DOPC in the presence of various concentrations of hexane reveal that at 22.5 °C up to 1 mol of hexane per mol of lipid is incorporated into the bilayer with little or no change in the density of the hydrocarbon region[17] and that the hexane is found intermingled with lipid acyl chains in the central 10 Å of the bilayer regardless of its concentration in the bilayer.

The ^{2}H NMR results presented above reval that the motional characteristics of hexane dissolved in liquid crystalline and gel phase DOPC bilayers are quite different. The high-temperature disordered phase imposes constraints on the hexane motional freedom resulting in anisotropic motion giving rise to the observed powder pattern line shape in the ^{2}H NMR spectra. In the more ordered gel phase the principal feature of the spectra is a single resonance in the center of the spectra. This indicates that in the gel phase hexane is undergoing isotropic motion. We outline two mechanisms by which this could take place. The first mechanism involves rotational diffusion of the hexane molecules within the gel phase. In effect they no longer sense the presence of the surrounding anisotropic media. This is a plausible mechanism considering that the order parameter of the hexane in the liquid crystalline bilayer is small ($\Delta\nu_q$ never larger than 10 kHz) and

(12) Derbyshire, W.; Corvin, T. C.; Warner, D. *Mol. Phys.* **1969**, *17*, 401–407.

(13) Burnett, L. J.; Muller, B. H. *J. Chem. Phys.* **1971**, *55*, 5829–5831.

(14) Kowalewski, J.; Lindblom, T.; Vestin, R.; Drakenberg, T. *Mol. Phys.* **1976**, *31*, 1669–1676.

(15) Seelig, S.; Seelig, J. *Biochemistry* **1977**, *16*, 45–50.

(16) Seelig, J.; Waespe-Sarcevic, N. *Biochemistry* **1978**, *17*, 3310–3315.

(17) King, G. I.; Jacobs, R. E.; White, S. H. manuscript in preparation.

that the shape of the molecule is probably not far from spherical (length to width ratio of *all-trans*-hexane is only 2.4). The second mechanism involves rapid lateral diffusion of hexane through different bilayer orientations with respect to the external magnetic field. When the correlation time for lateral diffusion (τ_c) becomes small enough that $2\pi\Delta\nu_q\tau_c \lesssim 1$, the ^{2}H NMR spectrum will exhibit isotropic behavior.[18] The correlation time is related to the radius of the liposome (r) and the diffusion constant of hexane in the liposome (D): $\tau_c = r^2/(6D)$. If $\Delta\nu_q = 10$ kHz and $D = 10^{-6}-10^{-18}$ cm^2 s^{-1} (on the order of the lateral diffusion constants observed for lipids in lipid bilayers[19]), then $r \lesssim 500-5000$ Å. Electron microscopy studies of multilamellar dispersions like those employed in this study have shown that they are complex highly convoluted structures containing large numbers of liposomes with radii in this range.[20,21] Lateral diffusion of the lipids in multilamellar dispersions accounts for the magnetization transfer across ^{2}H NMR powder pattern spectra observed in T_1 relaxation experiments.[22] Thus, even with the stated restrictions on D and r, lateral diffusion is a plausible mechanism to account for the observed isotropic behavior. We note that these two mechanisms are not mutually exclusive and that increasing the concentration of hexane in the bilayer would tend to increase both the translational and rotational diffusion constants toward those found in the pure alkane.

In POPC bilayers where the oleoyl chain is deuterated at the 9'- and 10'-carbons (i.e., the double bond) the ^{2}H NMR spectrum consists of two powder pattern doublets: one double from each deuteron.[15,16] In the present study we have examined DOPC bilayers in which both acyl chains have been deuterated at their double bond. We observe four overlapping powder patterns. The $\Delta\nu_q$'s for two of the power patterns are essentially the same as those found in the POPC system at the same reduced temperature (approximately 2.5 and 14.5 kHz, see Figures 4 and 6). One of the other quadrupole splittings is about halfway between the two POPC values (7.5 kHz). The remaining quadrupole splitting is similar to the larger of the two POPC values (14 kHz). Extrapolating from the POPC case, we conclude that each of the four powder patterns in the ^{2}H NMR spectra of double bond deuterated DOPC bilayers arises from one of the four deuterons. Further, it seems unlikely that the similarity between the POPC quadrupole splitting and one pair of the DOPC quadrupole

splittings mentioned above is coincidental. Therefore, we conclude that the order parameter for one of the double bonds in DOPC is the same as that for the double bond in POPC. To determine whether the different quadrupole splitting of the other double bond implies a different order parameter for that double bond, we calculated the deviation of the C=C bond vector from the bilayer normal and S_{mol} (the principal element of the order parameter tensor). S_{mol} is the apppropriate order parameter in this situation since it is free of all geometric effects. We used the method and assumptions employed by Seeling and Waespe-Sarcevic.[16] The most suspect assumption in this calculation is the value of S_{33} (0.36), which was measured for egg yolk lecithin bilayers[23]—a highly heterogeneous system. Using this value for S_{33}, we found that neither double bond is more than 10° from the bilayer normal and that $S_{mol} = 0.37$ for both of the DOPC double bonds (the same S_{mol} found for the POPC double bond[16]). It is clear from these calculations that the gross features of the motional characteristics of *all* of the double bonds in the DOPC and POPC bilayers are quite similar.

In conclusion, we have found that hexane dissolved in a pure lipid bilayer is a conceptually simple two-component system which exhibits some surprising characteristics. The bilayer will incorporate large quantities of the alkane. The phase behavior of the system is dependent upon the particular lipid involved, but in both cases examined the order of the lipid chains (as determined by ^{2}H NMR) is essentially unaffected by the presence of hexane. The anisotropy of the bilayer is reflected in anisotropic behavior of the dissolved alkane only at high temperatures (relative to the bilayer T_m) and relatively low concentrations. At low temperatures and high concentrations hexane undergoes isotropic motion within the bilayer, probably via increased rotational and/or translational diffusion within the bilayer. These results emphasize the complexity to be found in simple multicomponent lipid bilayer systems.

(18) Burnell, E. E.; Cullis, P. R.; de Kruijff, B. *Biochim. Biophys. Acta* **1980**, *603*, 63–69.

(19) Kuo, A.-l.; Wade, C. G. *Chem. Phys. Lipids* **1979**, *25*, 135–139.

(20) Hauser, H.; Phillips, M. C.; Stubs, M. *Nature (London)* **1972**, *239*, 342.

(21) Papahadjopoulos, D.; Miller, N. *Biochim. Biophys. Acta* **1967**, *135*, 624.

(22) Brown, M. F.; Davis, J. H. *Chem. Phys. Lett.* **1981**, *79*, 431–435.

(23) Fringeli, U. P. *Z. Naturforsch., C: Biochem., Biophys., Biol., Virol.* **1977**, *C32*, 20–45.