DEUTERIUM NMR SPECTROSCOPY OF SATURATED AND POLYUNSATURATED LIPID BILAYERS

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1. INTRODUCTORY REMARKS

Living organisms are composed of cells and cells are composed of membranes -- thus the study of membranes is vital to the study of life itself. It is known that membranes comprise proteins mediating distinctive biological functions, embedded in or bound to a bilayer of lipid molecules which provides a permeability barrier, and may influence function through lipid-protein interactions (Stryer, 1981; Wiedmann et al., 1988). Yet many fundamental questions remained unanswered. Which properties of membrane lipid bilayers influence biological functions and how can these be studied by appropriate physicochemical methods? How are structural and dynamic properties of membraneous lipids in the liquid-crystalline state related to their polar head groups and the length and degree of unsaturation of their fatty acyl chains? Do common principles explain the properties of liquid crystals, lipid bilayers, and biomembranes?

2. SATURATED AND POLYUNSATURATED PHOSPHOLIPID BILAYERS STUDIED BY DEUTERIUM NMR SPECTROSCOPY

We have recently carried out $^2$H NMR studies of various phosphatidylcholines in the liquid-crystalline ($L_\alpha$) state, in which influences of the chemical length and polyunsaturation of the acyl chains on membrane properties have been investigated (Fig. 1). These studies are of fundamental interest since the nature of the acyl chains and polar head groups of phospholipids appears related to the effective bilayer thickness and chain cross-sectional area (Gruner et al., 1985; Salmon et al., 1987). There is evidence that such properties may be important with regard to biological functions carried out by membrane proteins (Wiedmann et al., 1988).
Disaturated Phosphatidylcholines. Fig. 2 shows $^2\text{H}$ NMR spectra obtained for a homologous series of 1,2-diacyl-sn-glycero-3-phosphocholines with saturated, perdeuterated chains ranging from $n = 12$ to 16 carbon atoms [abbreviated $\text{di(per-}^{2}\text{H-}n\text{-}0\text{)PC}$]. The $^2\text{H}$ NMR spectra were numerically deconvolved by a procedure called de-Pakeing (Pauls et al., 1983). A number of individually resolved splittings are evident, which arise from interaction of the quadrupole moment of the $^2\text{H}$ nucleus with the electrostatic field gradient of the C-$^2\text{H}$ bond. The separation in frequency units between each pair of lines is related to what is called the order parameter of the corresponding C-$^2\text{H}$ labeled segment by (Seelig and Seelig, 1980)

$$S_{CD}^{(i)} = 1/2 < 3 \cos^2 \beta_i - 1 >. \quad (1)$$

In the above expression $\beta_i$ is the instantaneous angle between the $i$th segmental C-$^2\text{H}$ bond and the perpendicular to the bilayer surface, known as the director axis; the brackets indicate a time or ensemble average. For an all-trans chain, the absolute value of $S_{CD}^{(i)}$ equals 1/2, which is reduced in the liquid-crystalline state by trans-gauche isomerizations together with any whole molecule motions. The largest splittings in parts a-c of Fig. 2 are from those C$^2\text{H}_2$ groups nearest the ester carbonyl moiety, with a progressive reduction along the chains until the C$^2\text{H}_3$ termini are reached, which yield the smallest splittings in the center of the spectra. Thus one is seeing the profile of the local ordering along the chains at the atomic level. The $S_{CD}^{(i)}$ values can be plotted...
as a function of the chain index $i$ to obtain an order profile which is characteristic of the liquid-crystalline state (Pauls et al., 1983; Salmon et al., 1987). Since the greatest spectral intensity corresponds to the largest quadrupolar splittings (cf. Fig. 2), a "plateau" in the distribution of the order parameters as a function of chain position exists, which reflects packing of the acyl chains in the bilayer hydrocarbon region (Seelig and Seelig, 1980).

Fig. 2. Deuterium $^2$H NMR spectra of a homologous series of disaturated phosphatidylcholines with perdeuterated acyl chains. The spectra were numerically deconvoluted (de-Paked) and correspond to the $\theta = 0^\circ$ orientation of the bilayer normal $n_\alpha$ relative to the main magnetic field $B_0$. All samples contained 50 wt. % H$_2$O and were in the $L_\alpha$ phase at 50°C.

Fig. 3. De-Paked $^2$H NMR spectra of a homologous series of mixed chain, polyunsaturated phosphatidylcholines. The $sn$-1 saturated chains are perdeuterated, whereas the $sn$-2 polyunsaturated chain (22:6ω3) is protiated. All samples contained 50 wt. % H$_2$O and were in the $L_\alpha$ phase.
The de-Paked $^2$H NMR spectra (Fig. 2 a-c) show rather clearly that the relative intensity due to the components with largest quadrupolar splittings increases with the number of chain carbon atoms from $n = 12$ to 16 (and to 18 carbons; not shown). By contrast, the splitting due to the terminal $C^2H_2$ group and the seven splittings resolved in the central region of the spectra remain almost constant. Thus, the length of the plateau in the order profile increases as the number of carbon atoms in the chains increases at the same absolute temperature. In the liquid-crystalline state, trans-gauche isomerizations of the chains lead to a shortening of their effective lengths projected along the bilayer normal. Since a statistical distribution of trans-gauche isomers exists, one has a distribution of projected lengths. The average length $<L>$ and the increment to $<L>$ per methylene group can be calculated using the expression (Salmon et al., 1987)

$$<L> = 1 \left[ \frac{n}{2} - \sum_{i=1}^{n-1} S_{CD}^{(i)} - 3S_{CD}^{(n)} \right]. \quad (2)$$

Here $l = 1.25$ Å and $i = 1, ..., n$ denotes the fatty acyl chain segments counting from the $C^2H_2$ group adjacent (alpha) to the carbonyl moiety ($i = 1$) to the terminal $C^2H_3$ group ($i = n$). The conclusion is that the average physical length of the chains projected along the bilayer normal increases with the number of chain methylene groups; that is, the additional mass goes to increase the bilayer thickness rather than its surface area (cf. Lewis and Engelman, 1983). Thus the cross-sectional area per molecule is governed mainly by interactions among the polar headgroups, which modulate indirectly the interactions between the chains. A value for the isobaric thermal expansion coefficient $\alpha = 1/<L>$ $(\partial<L>/\partial T)_P = -1.5 \times 10^{-3}$ K$^{-1}$ is obtained from the temperature dependence of the order parameters, which appears independent of the number of acyl chain carbon atoms.

**Mixed-Chain Saturated-Polyunsaturated Phosphatidylcholines.** A series of comparative investigations of polyunsaturated phosphatidylcholines in the $L_\alpha$ phase has also been carried out (cf. Fig. 1). The phosphatidylcholines contain a saturated, perdeuterated acyl chain ranging from 12 to 18 carbons at the glycerol sn-1 position, and a polyunsaturated, protiated chain comprising 22 carbons and 6 double bonds at the sn-2 position [abbreviated (per-$^2$H-$n$:0)(22:6)PC, where $n = 12, 14, 16, \text{and } 18$]. Thus the saturated chain is $^2$H-labeled, and one is interested in whether its properties differ from those of the corresponding disaturated phospholipids due to esterification next to the 22:6
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chain. The polyunsaturated fatty acid is docosahexaenoic acid, which is a major constituent of visual membranes as well as dietary fish oils thought to be of benefit with regard to atherosclerosis. De-Paked $^2$H NMR spectra of the series of mixed-chain, polyunsaturated phosphatidylcholines in the L$_\alpha$ phase are shown in Fig. 3. The $^2$H NMR spectra differ from the corresponding disaturated phosphatidylcholines (Fig. 2) in that the largest splittings have less intensity and additional components with intermediate splittings are evident. Hence the plateau in the order profiles is significantly shorter than for the corresponding disaturated phosphatidylcholines (Salmon et al., 1987). As the saturated chain is increased progressively from 12 to 18 carbons, the resolved quadrupolar splittings decrease and new splittings appear, reflecting the increased number of chain deuteromethylene groups.

The shorter order profiles and increased number of intermediate splittings suggest that the saturated chains have greater configurational freedom when esterified next to a 22:6 chain than in disaturated phospholipids. For the entire series, the effective length $<L>$ and the increment per methylene group are significantly less than for the corresponding disaturated phosphatidylcholines. Consequently the balance of opposing attractive and repulsive forces may be such that the chain packing is somewhat looser in the former. The polyunsaturated bilayers may be slightly thinner due to compaction of the 22:6 chains along the bilayer normal, perhaps due to helical conformations or interdigitation of the saturated and polyunsaturated chains (Salmon et al., 1987). It is thought that the balance of forces within the head group and hydrocarbon chain regions is related to the polymorphic phase behavior of lipids (Gruner et al., 1985). As a result, the increased chain freedom detected with $^2$H NMR may indicate properties favoring the inverted hexagonal (H$_{II}$) phase at lower temperatures than for the disaturated phosphatidylcholines.

3. MOLECULAR DYNAMICS AND NUCLEAR SPIN RELAXATION OF LIPID BILAYERS

In addition to average properties obtained from the $S_{CD}^{(i)}$ values, the corresponding spin-lattice relaxation rates $R_{1z}^{(i)}$ yield a second quantity related to the dynamics of the lipid molecules within the bilayer. The $R_{1z}^{(i)}$ rates of lipid bilayers depend on both the amplitudes and rates of the molecular motions. The amplitude is in general characterized by the order parameter $S_{CD}^{(i)}$, whereas the rate of the segmental fluctuations is described by one or more correlation times (like a memory time of the prior orientation). Thus a combined
analysis of the $R_{1z}^{(i)}$ rates and $S_{CD}^{(i)}$ values can provide insight regarding the molecular dynamics of lipid bilayers over a range of different time-scales (Brown et al., 1979; Brown, 1982; Jarrell et al., 1988; Pastor et al., 1988). In what follows, our attention will be confined to the bilayer hydrocarbon region.

Fig. 4 shows profiles of the $R_{1z}^{(i)}$ rates and $S_{CD}^{(i)}$ values as a function of chain position for a representative disaturated bilayer, di(per-$^2$H-12:0)PC, and a representative mixed-chain polyunsaturated bilayer, (per-$^2$H-12:0)(22:6)PC, in the liquid-crystalline state. In both cases, the relaxation profile parallels the order profile in that a plateau is observed over the first part of the chain, followed by a decrease towards the chain terminus. In other words, we have two functions -- a relaxation function and an order function -- which vary with the carbon segment position. Since we know the relaxation function depends on the order function, what is the nature of the dependence of the $R_{1z}^{(i)}$ values on the corresponding values of $S_{CD}^{(i)}$? In other words, how can we separate the static and dynamic information contained in the relaxation profiles?

![Fig. 4. Profiles of the order parameters $S_{CD}^{(i)}$ and spin-lattice relaxation rates $R_{1z}^{(i)}$ as a function of chain segment position. Representative data are shown in parts a and b, respectively, for the disaturated and mixed-chain polyunsaturated phosphatidylcholines in the Lα phase at 30°C. The two $^2$H-labeled chains are inequivalent in the case of the disaturated bilayer, leading to different order parameters.](image)

Experimentally, one observes that the relaxation profile $R_{1z}^{(i)}$ often depends on the square of the order profile $S_{CD}^{(i)}$. That is to say, if we take the two quantities measured for each of the resolved splittings, namely $R_{1z}^{(i)}$ and $S_{CD}^{(i)}$, and then plot the $R_{1z}^{(i)}$ values versus the corresponding $S_{CD}^{(i)}$ values, a straight line is obtained (Brown, 1982; Williams et al., 1985). Representative plots are shown in Fig. 5 for the disaturated and mixed-chain polyunsaturated bilayers. The above observation is independent of any model to interpret the findings -- it is simply phenomenological in nature. Is there an underlying physical meaning to the observed square-law functional dependence?
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One explanation is that the observed $R_{1z}^{(i)}$ rates detect the presence of multiple motions of the hydrocarbon chains within the bilayer, including fast, local motions as in liquid paraffins, together with slower disturbances reflecting the chain packing (Brown, 1982; Brown et al., 1983; Brown and Williams, 1985). The fast motions represent fluctuations of the C-2H segments with respect to a local director axis, such as trans-gauche isomerizations and rotational diffusion of the chains. Slower motions due to cooperative chain reorientations can then lead to displacements of the instantaneous director with respect to the average director or bilayer normal. Since the slow motions affect the ordering set-up or left-over by the fast motions, they are referred to as order-director fluctuations (ODF). The observed relaxation rate is given by the sum of the fast and slow contributions, leading to the result that (Brown, 1982)

$$[R_{1z}^{(i)}]_{obs} = [R_{1z}^{(i)}]_{fast} + [R_{1z}^{(i)}]_{slow},$$

(3)

$$= A \tau_f^{(i)} + B^{(i)} |S_{CD}^{(i)}|^2 f(\omega_0).$$

(4)

In the above, $A$ and $B^{(i)}$ are constants and $\tau_f^{(i)}$ is the correlation time for fast motions of the $i$th segment, which is related to the effective or microviscosity $\eta$ of the bilayer by $\tau_f^{(i)} = V_{CH2}^{(i)} \eta/kT$; $k$ is the familiar Boltzmann constant and $V_{CH2}^{(i)}$ is an effective volume. The observed segmental order parameters are then given by $[S_{CD}^{(i)}]_{obs} = S_{slow}^{(i)} [S_{CD}^{(i)}]_{fast}$, where $S_{slow}^{(i)}$ is an order parameter describing the amplitude of the slow motions and $[S_{CD}^{(i)}]_{fast}$ corresponds to fast motions such as trans-gauche isomerizations. The functional form of the slow motional contribution (that is the spectral density) is described by $f(\omega_0)$, where $\omega_0$ is the resonance frequency of the $^2$H nucleus (proportional to the magnetic field strength). Recent results suggest that an $\omega_0^{-1/2}$ dependence is appropriate.

The fact that a square-law correlation is predicted by the simple ODF model suggests, but does not prove, that such motions exist in lipid bilayers and are important in determining the relaxation under the experimental conditions of temperature and magnetic field strength (frequency). Within the context of the model employed, the slope of the square-law plots is related to the amplitude of the slow motions as described by $S_{slow}^{(i)}$, in addition to other quantities that will recognized by the connoisseur of relaxation, but for reason of brevity not discussed here (cf. Brown, 1982). Since straight lines are typically (but not always) obtained, the parameters $\tau_f^{(i)}$ and $B^{(i)}$ of the ODF model do not depend strongly on the segment position $i$ to a first approximation. For a collective
model, involving a continuous distribution of correlation times, the amplitude of the slow motions as described by $S_{\text{slow}}^{(i)}$ cannot be estimated at present. But assuming a noncollective model, a value of $S_{\text{slow}}^{(i)} = 0.7$ is obtained (Brown, 1984). The observed order parameters $[S_{\text{CD}}^{(i)}]_{\text{obs}}$ would thus appear to represent a lower limit to the local order parameters $[S_{\text{CD}}^{(i)}]_{\text{fast}}$ describing rotational isomerization of the chains. Since the square-law plots for the disaturated and mixed-chain polyunsaturated bilayers are largely superimposable, the coefficients $b^{(i)} = B$ do not differ appreciably, suggesting that the amplitude of the slow motions is similar in the two cases. This finding is in contrast to a recent report by Paddy et al. (1985). Thus the differences in the $^2$H NMR spectra and derived order profiles of the disaturated and mixed-chain polyunsaturated bilayers appear largely due to faster motions such as trans-gauche isomerizations.

What about the rates of the local chain motions of the disaturated and mixed-chain polyunsaturated bilayers? For the model employed, the intercept value of $R_{1z}^{(i)}$ obtained by extrapolating the square-law plots to zero ordering is related to the segmental correlation times $\tau_f^{(i)}$, which in turn are related to the effective or microviscosity of the bilayer hydrocarbon region (see above). The data suggest that the microviscosity of a bilayer, where a bulk viscosity cannot be measured, corresponds roughly to that of a liquid n-paraffin having a bulk viscosity of about 1-2 cP. Similar results are obtained for the disaturated and polyunsaturated bilayers (cf. Fig. 5). Due to the lower-order disorder transition temperatures, the latter are often assumed to be more "fluid" which does not appear to be the case. This interpretation (Brown, 1982; Brown et al., 1983) differs from previous fluorescence depolarization studies of extrinsic

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Fig. 5. Plots of the spin-lattice relaxation rates $R_{1z}^{(i)}$ versus the square of the corresponding order parameters $S_{\text{CD}}^{(i)}$. Representative data are shown for the sn-1 chains of the disaturated (□) and mixed-chain polyunsaturated (○) bilayers in the L-α phase at 15°C.
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probe molecules in lipid bilayers (Shinitzky and Barenholz, 1978), but is now supported by recent Brownian dynamics simulations (Pastor et al., 1988). Needless to say, it is unlikely that a single microviscosity parameter can adequately describe the molecular dynamics of lipid bilayers in the liquid-crystalline state. Finally, the relaxation behavior of the membrane phospholipid glycerol backbone and phosphocholine head group segments may differ from the hydrocarbon chains and merits further study.

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