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Two Pictures of a Lipid Bilayer. A Comparison between Deuterium Label and Spin-Label Experiments†

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ABSTRACT: The segmental order parameters in a lipid bilayer are measured by means of deuterium magnetic resonance and by means of spin-labels. The deuterium results show an almost constant order parameter over most of the chain, which decreases rapidly near the last three carbon atoms. In contrast, the spin probes reveal a continuous decrease of the order parameter. The deuterium results show the physical state of the unperturbed bilayer. They can be interpreted in terms of a kink model for the bilayer structure. The spin-labels reveal the response of the bilayer on a small perturbation.

During the last 5 years lipid bilayers have been studied extensively by means of spin probes (*cf.* review by Jost *et al.* (1971)). Deuterium magnetic resonance (dmr) of selectively deuterated lipid samples provides similar structural information but with the advantage of not perturbing the liquid crystalline phase (Seelig and Niederberger, 1974). Our previous report dealt with some methodological aspects of deuterium labeling. We present here the results of a lipid bilayer study including both deuterium labels and spin-labels. Two different pictures are obtained for the structure of the lipid bilayer depending on the method used.

Experimental Section

The bilayer system was a liquid crystalline phase of the following chemical composition: sodium decanoate (32 wt %), decanol (38 wt %), and water (30 wt %), where the decanoic acid was replaced by its selectively deuterated analog. Nine bilayer phases of the above mentioned lipid composition were prepared, each containing a CD_2 group (CD_3 for the last C atom) at a different position in the hydrocarbon chain of the decanoic acid. In all samples, the decanol molecule was replaced by 1,1-dideuteriodecanol, so that the residual quadrupole splitting of this alcohol could be used as an internal standard. The deuterated substances were synthetized by Kolbe electrolysis (Nguyên-Dinh-Nguyên, 1968).

The bilayer phase (400 mg) was prepared in a sealed ampoule and placed in a 10-mm nuclear magnetic resonance (nmr) tube. The dmr measurements were performed at ambient temperature (~28°) with a Bruker FFT-HX 90-spectrometer operating at 13.8 MHz. Our previous instrumentation was improved by a Bruker BNC-73 computer with a spectral range of 50 kHz (compared to 25 kHz in the earlier experiments). A 90° pulse of 18 μsec width was used. In the liquid crystalline bilayer the deuterium nucleus has a rather short T_1 relaxation time, so that 9000 free induction decays could be accumulated in 30 min.

Results

Typical dmr spectra are shown in Figure 1. The molar ratio decanol–sodium decanoate in this bilayer is about 1.5, and therefore the more intense signal is due to 1,1-dideuteriodecanol. The smaller peak arises from deuterated decanoic acid. The residual quadrupole splitting $\Delta\nu_p$ (= separation of the corresponding doublets) is constant for 1,1-dideuteriodecanol, but changes for the decanoic acid depending on the position of the deuterium in the chain. The residual quadrupole splittings are listed in Table I. The experimental error amounts to approximately 0.5–3%. From the residual quadrupole splittings the order parameter of the deuterium bond S_{CD} and that of the chain segment S_{mol} can be calculated as described previously (Seelig and Niederberger, 1974). These results are also given in Table I. A quadrupole constant of 170 kHz, characteristic of deuterated paraffins (Burnett and Muller, 1971), was used for all CD bonds regardless of the position in the chain.

Following the dmr experiments the bilayer phases were doped with a small amount of stearic acid spin probes. The spin probes and the experimental procedures have been described previously (Seelig, 1970; Seelig *et al.*, 1972). The order parameter S_3 measures the average orientation of the chain segment at the position of the NO group. In principle, both order parameters S_{mol} and S_3 should have the same value for a given chain segment.[1] The spin-label results are listed in the last column of Table I.

Discussion

In Figure 2 we have plotted the order parameters S_{mol} and S_3 as a function of the chain position. There is a distinct

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[1] Deuterium magnetic resonance is sensitive to slower motions than electron spin resonance. This plays no role in our system but may be important for phospholipids as has been pointed out to us by Professor H. M. McConnell.

TABLE I: Experimental Quadrupole Splittings and Order Parameters.

		Deuterium Labels			Spin-Labels
Labeled Carbon Atom No.	Labeled Compd	Quadrupole Splitting $\Delta\nu_p$ [kHz]	Order Parameters S_{CD}	Order Parameters S_{mol}	Order Parameter S_3
1	Decanol	28.3 ± 0.15	−0.222	0.444	
2	Decanoic acid	36.3 ± 0.5	−0.285	0.570	
3	Decanoic acid	35.1 ± 0.5	−0.275	0.550	
4	Decanoic acid	37.6 ± 0.5	−0.295	0.590	0.62
5	Decanoic acid	38.1 ± 0.5	−0.299	0.598	0.58
6	Decanoic acid	36.5 ± 0.5	−0.286	0.572	0.50
7	Decanoic acid	36.7 ± 0.5	−0.288	0.576	0.43
8	Decanoic acid	30.6 ± 0.4	−0.240	0.480	0.35
9	Decanoic acid	24.1 ± 0.4	−0.189	0.378	0.25
10	Decanoic acid	8.4 ± 0.2	−0.066	0.198	0.21

difference between the two label experiments. The deuterium labels show an almost *constant* order parameter over most of the chain, which decreases rapidly near the last three carbon atoms. In contrast, the spin probes reveal a *continuous decrease* of the order parameter. (In an independent study Charvolin *et al.* (1973) have detected a *constant* deuterium order parameter for the first nine segments of a bilayer composed of perdeuterated potassium laureate. No spin-label studies were performed with this system.)

We attribute the discrepancy between the spin-label and the deuterium-label experiments to the perturbing influence of the nitroxide group. This is not meant to discriminate against spin-labels. Near the polar region the spin-label order parameter S_3 is almost equal to the deuterium order parameter S_{mol}. The bilayer therefore remains intact, although the chain conformation may be distorted. A comparable situation is probably the incorporation of a protein into a lipid membrane. Proteins are known to penetrate the lipid phase of membranes (Bretscher, 1973), and they may deform the neighboring lipid chains in a similar way as the nitroxide group. Both the deuterium-label and the spin-label method provide information of biological significance, but they reflect different aspects of the physical situation in a bilayer. The deuterium labels show the structure of the unperturbed bilayer, while the spin-labels detect the response of the bilayer under a small physical stress.

The current picture of a lipid bilayer has been strongly influenced by the spin-label experiments. The model used to interpret the decrease of the order parameter S_3 can be described as follows (Seelig, 1970, 1971; Hubbell and McConnell, 1971). Each carbon–carbon bond is assumed to have a certain intrinsic flexibility, due to rotational isomerizations between trans (t) and gauche (g^+, g^-) conformations. One end of the chains is anchored in the lipid–water interface and thus fixed. The other end is free to move. The overall motion is determined by the superpositions of segmental motions. Near the polar surface the motional freedom is rather restricted since only a few chain segments are involved. The motion becomes increasingly more random toward the central part of the bilayer due to the increasing number of flexible CH_2 groups. The lipid bilayer has therefore been described as *ordered* near the polar region and *disordered* in the interior. (The terms *solid* and *fluid* which are also invoked to characterize this structure are misleading. The rate of the anisotropic rotation near the polar region is almost equal to that in the central part (Schindler and Seelig, 1973).)

The conceptual difficulties inherent to the spin-label model are the following. The model describes the properties of *individual* chains. The interactions between adjacent chains are taken into account by an apparent increase in the energy of the gauche conformation, but otherwise the chains are considered as completely independent of each other. The model does not explain how the lipid molecules are packed in the bilayer.

The deuterium labels suggest an alternative picture for the lipid bilayer. The experimental data cannot be interpreted by the spin-label model, but require conformational changes

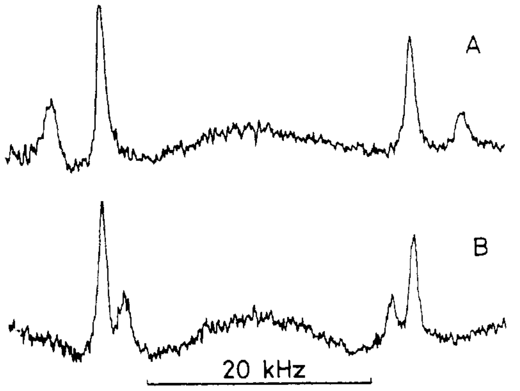

FIGURE 1: Deuterium magnetic resonance spectra of selectively deuterated lipid bilayers. Phase composition: (A) 1,1-dideuteriodecanol (38 wt %), sodium 5,5-dideuteriodecanoate (32 wt %), water (30 wt %); (B) 1,1-dideuteriodecanol (38 wt %), sodium 9,9-dideuteriodecanoate (32 wt %), water (30 wt %).

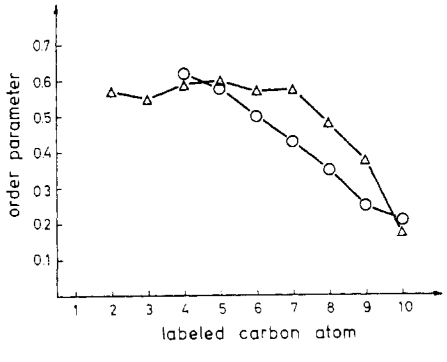

FIGURE 2: Segmental order parameter as a function of the label position: (○) spin-label order parameter S_3; (△) deuterium label order parameter S_{mol}.

which leave the hydrocarbon chains essentially parallel. The packing of the chains is then easily visualized, and the bilayer structure can be described as a defective-ordered structure (kink model).

Let us develop this concept starting from rather simple models for the lipid bilayer. We consider a hypothetical bilayer with the lipids frozen in the all-trans conformation and oriented perpendicular to the plane of the bilayer. The order parameters of all chain segments are then equal to 1. Next we generate small angular fluctuations of the stiff chains with the bilayer normal as the axis of motional averaging. This rigid body motion leads to a smaller order parameter S_o, where α

$$S_o = {}^1/_2(3\langle\cos^2 \alpha\rangle - 1) \qquad (1)$$

denotes the angular deviation from the bilayer normal. Again all chain segments have the same order parameter, since they experience the same angular fluctuations. However, stiff hydrocarbon chains are quite improbable in lipid bilayers. X-Ray diffraction studies of liquid crystalline bilayers show the diffuse reflection at $(4.6\ \text{Å})^{-1}$ typical of *liquid* paraffins (Luzzati, 1968; Levine and Wilkins, 1971; Ekwall *et al.*, 1968). We must therefore introduce conformational changes into our model. Two types of conformational changes are important for the interpretation of the order parameter: (1) the formation of an isolated gauche conformation ($tg^{\pm}t$) and (2) the formation of a kink (g^+tg^-, g^-tg^+). (One can imagine more sophisticated conformations but these are either highly improbable or can be constructed by a suitable combination of t, g^+tg^-, and $tg^{\pm}t$ sequences. They do not affect the qualitative conclusions described below.)

Let us first consider an isolated gauche conformation. The formation of a $tg^{\pm}t$ sequence entails the collective reorientation of all segments between the $g^{\pm}$ element and the terminal methyl group (*cf.* Figure 3). If the probability of the $tg^{\pm}t$ sequence is assumed to be constant throughout the chain this model leads to an exponential decrease of the order parameter. This is essentially the spin-label model and is inconsistent with the *constant* deuterium order parameter. Only the decrease of the deuterium order parameter close to the terminal methyl group could be explained by this model.

Next we investigate the effect of kink formation. A kink is defined as a g^+tg^- (g^-tg^+) sequence. Its prominent feature is a shift of a part of the molecule perpendicular to its long molecular axis, that is, trans sequences before and after the kink remain parallel to each other but are displaced on their lattice position by approximately 1.5 Å. At the same time the chain is shortened by one CH_2 unit. The formation of a kink at a fixed position in a trans chain has the following effect on the order parameters. The segments before and after the kink have the same order parameters, since their chain vectors are parallel to each other. At the kink itself two CH_2 units are bent by 60° with respect to the chain axis, thus reducing the order parameters of these segments by a factor $|S| = |{}^1/_2(3\cos^2 60° - 1)| = 0.12$. Next we assume that the kink is not localized at a certain chain segment, but is fluctuating up and down the chain. If the lifetime of an individual conformation is shorter than $\sim 10^{-6}$ sec and if the formation of a kink occurs with *equal probability* regardless of the chain position, this model leads to a *constant* order parameter for all chain segments.

The constant order parameter of the deuterated lipids is thus in agreement with the predictions of the kink model. This leads to the following picture for a *pure lipid* bilayer. The lipid molecules are aligned essentially parallel to each other. The interaction energy between adjacent chains is small enough so that the perfect parallel alignment can be interrupted by the formation of rapidly fluctuating kink defects. This probably explains the diffuse X-ray band at $(4.6\ \text{Å})^{-1}$. At the interface of the two half-layers the segmental motion shows an increasing disorder. The bilayer may therefore be described as a *defective ordered* structure (Träuble, 1971).

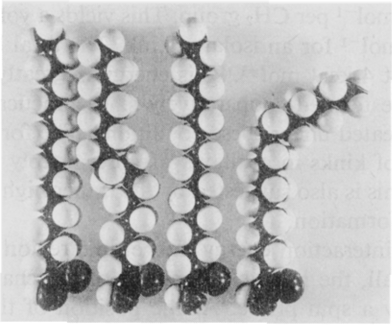

FIGURE 3: Molecular models of various chain conformations. All-trans conformation (1st and 3rd chain), kink (2nd chain), and isolated gauche conformation (4th chain).

A similar structure prevails in crystals and melts of polyethylene near the transition point. The kink model has provided a quantitative description of the elastic and thermodynamic properties of these systems (Pechhold, 1968; Blasenbrey and Pechhold, 1970). The formation of kinks in bimolecular films has been found by means of X-ray diffraction in intercalation compounds of layer silicates (Lagaly and Weiss, 1971). Based on the ideas of Pechhold the kink model has been applied to explain the permeation of neutral molecules through lipid bilayers (Träuble, 1971). The kink model has also been used to interpret ^{1}H nmr relaxation time measurements in sonicated lecithin dispersions (Horwitz *et al.*, 1972, 1973). Our deuterium results seem to be a rather direct experimental support of this model.

The probability of kinks, p_{kink}, in the unperturbed bilayer can be estimated as follows. If S_o is the order parameter of the rigid body motion and p_t the probability of a carbon–carbon bond being trans, then the experimental order parameter S_{mol} is given by

$$S_{\text{mol}} = S_o[p_t + p_{\text{kink}}(3\cos^2 60° - 1)] \qquad (2)$$

with

$$p_t + 2p_{\text{kink}} = 1 \qquad (3)$$

Unfortunately, the order parameter S_o is not known for lipid bilayers. A lower limit is probably $S_o \sim 0.7$, which is a typical value for nematic liquid crystals (Saupe, 1968). With $S_{\text{mol}} = 0.57$ one then obtains the following probabilities: $p_{\text{kink}} \approx 0.08$ and $p_t \approx 0.83$. This corresponds to approximately one kink fluctuating in the region of constant order parameter of our rather short bilayer. (For $S_o = 1$ the kink probability is $p_{kink} \approx 0.19$.) Neglecting entropy effects the formation of an *isolated* kink involves essentially two different energies (Blasenbrey and Pechhold, 1970): (1) the intramolecular energy $E_{\text{rot}} = 2 \times 0.5$ kcal mol^{-1} required to form two gauche states and (2) the change in the intermolecular energy due to the additional kink volume. For an isolated kink this volume corresponds to approximately 2.6 CH_2 groups. From studies of lipid monolayers (Gershfeld and Pagano, 1972) and from studies of polyethylene melts (Blasenbrey and Pechhold, 1970) the cohesion energy in the bilayer can be estimated to be 1 kcal mol^{-1} per CH_2 group. This yields a volume energy of 2.6 kcal mol^{-1} for an isolated kink. The total kink energy is thus about 4 kcal mol^{-1}. This energy is greatly reduced if the kinks are formed cooperatively since practically no new volume is created under these conditions. The formation and the motion of kinks in a bilayer are presumably cooperative processes. This is also suggested by the rather high probability of the kink formation.

Since the interaction energy in the lipid region of a bilayer is rather small, the parallel alignment of the chains is easily perturbed by a spin probe. At the position of the nitroxide group the cross section of a fatty acid spin probe is approximately twice that of a normal chain. If such a molecule is then incorporated into a bilayer, the neighboring chains are pushed apart by the NO protuberance and a large defect volume is created. This free volume facilitates the formation of gauche sequences and would explain the decrease of the spin-label order parameter S_3. The incremental decrease of S_3 is directly related to the average interaction energy in the bilayer (Limacher and Seelig, 1972) and a value of 0.8–1.0 kcal mol^{-1} per CH_2 unit is found. The spin–labels also indicate that this interaction energy is constant in most parts of the bilayer. These results of the spin-label method are therefore in agreement with the deuterium experiments.

Our dmr measurements refer to a rather simple bilayer, but the above bilayer picture is probably typical also for phospholipid bilayers. Measuring ^1H nmr relaxation times of lecithin dispersions several researchers have concluded a uniform motion or a rather shallow increase of motional freedom for most fatty acid methylene protons and an abrupt increase of motional freedom very close to the terminal methyl group (Horwitz *et al.*, 1972; Chan *et al.*, 1972). These results may be at variance with the spin-label results of the same systems, but since the molecular interpretation of relaxation times in anisotropic systems is extremely difficult and subjected to much larger errors than, for example, the evaluation of dmr or spin-label spectra, this issue is not yet settled. We are therefore synthetizing specifically deuterated L-α-dipalmitoyllecithins to decide this question.

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