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Effect of a Single Cis Double Bond on the Structure of a Phospholipid Bilayer†

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ABSTRACT: The ordering of the hydrocarbon chains and the rates of lipid motion are two independent parameters characterizing the structure and the dynamics, respectively, of a bilayer membrane. In this work, deuterium magnetic resonance has been used to elucidate the influence of a single cis double bond on the hydrocarbon chain *ordering* of a phospholipid bilayer. 1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine was specifically deuterated at various segments of the palmitic acyl chain and at the 9, 10 position of the oleic acyl chain, and the segmental order parameters were deduced from the quadrupole splittings of the unsonicated bilayer phases. The shape of the order profile of the palmitic acyl chain is similar to that observed for the corresponding fully saturated membrane, but the magnitude of the order parameters is distinctly smaller in the unsaturated system. This demonstrates that the presence of a double bond in a membrane causes a more disordered conformation of the hydrocarbon chains. Considering the *relative* flexibility within the palmitic acyl chain, the deuterium resonance data indicate a local stiffening of those segments which are located in the vicinity of the double bond. The membrane *fluidity* was investigated using a nitroxide-labeled stearic acid spin probe. The smaller electron paramagnetic resonance line width in bilayers of 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine demonstrates an increased fluidity compared to bilayers of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine.

The phospholipids found in bacterial and mammalian cell membranes usually contain saturated as well as unsaturated fatty acyl chains. There is a strong positional preference of the two types of fatty acids, with the saturated and unsaturated substituents being localized at position 1 and 2, respectively, of the glycerol part of the molecule. Monounsaturated phospholipids are predominant but lipids containing more than one double bond also occur quite commonly. The incorporation of a double bond introduces a rigid element into the otherwise flexible hydrocarbon chain and, furthermore, since double bonds are usually found to be in the cis conformation, it prevents the chain from assuming a completely extended configuration. Double bonds are important in biological membranes for at least two reasons. Firstly, it is known that the activity of certain membrane-bound enzymes depends critically on the presence of cis double bonds (Rothfield and Romeo, 1971), indicating a specific interaction between enzymatic recognition sites and the cis double bond. Since the replacement of the unsaturated grouping by a *cis*-cyclopropane ring also leads to almost equally active enzymes, it may further be inferred that in these cases the cis double bond plays its activating role not by its chemical nature but by its unique configuration. The second effect of cis double bonds is to control the physiological properties of membranes by a nonspecific, purely thermodynamic mechanism, namely, by modulating the membrane "fluidity". In the case of the bacterium *Escherichia coli,* it has been demonstrated quite convincingly that the proper functioning of the system requires the membrane lipids to be in the "fluid" (liquid crystalline) state (Overath et al., 1970, 1975). The enzymatic activity of membrane-bound proteins is greatly slowed down if the bacterium is cooled to temperatures where the lipids become rigid. It is known from thermal studies with synthetic lipids that the incorporation of cis double bonds into a saturated lipid drastically lowers the gel-to-liquid crystal transition point (Ladbrooke and Chapman, 1969; Phillips et al., 1972), which has been interpreted to suggest that the probability of a membrane to be "fluid" increases with the percentage of double bonds. This has indeed been verified experimentally for *E. coli* membranes using appropriate *E. coli* fatty acid auxotrophs (Overath et al., 1971).

The notion of a "fluid" membrane is, however, not sufficient

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for a characterization of the physical state of the membrane. It can be expected that differences exist between a "fluid" saturated and a "fluid" unsaturated bilayer, since at a given temperature the unsaturated bilayer system is usually much higher above its gel-to-liquid crystal transition point than is the saturated one. It is for the latter reason that bilayers with double bonds are often described as being more "fluid" than the corresponding fully saturated bilayers at the same temperature. Beyond simply referring to the gel-to-liquid crystal transition point (if at all measurable), a comparative study of membrane "fluidity" is rather difficult. One criterion for a "fluid" membrane is the observation of a diffuse x-ray reflection at $(4.6\ \text{Å})^{-1}$ arising from the lateral packing of the chains and representing some average value for the interchain separation. The marked similarity of this band with that of liquid paraffins leads to the conclusion that the fatty acyl chains in a bilayer must be visualized as highly *disordered*, like in liquid paraffins (Tardieu et al., 1973). Other measures of membrane "fluidity" are the proton and carbon-13 relaxation times of phospholipid bilayers and the EPR[1] line width of intercalated nitroxide spin probes. From the EPR and NMR studies published so far, it is apparent that the lipid molecules in a membrane undergo various types of *rapid motions*. Thus, there are at least two different parameters involved in membrane "fluidity", namely, (1) the *ordering* of the hydrocarbon chains (average configuration) and (2) the *rates* of the different types of motion (segment rotation, lateral diffusion). These two parameters, order and rate, are, to a certain extent, independent of each other (Seelig and Seelig, 1974). In a more restricted sense, the term "fluid" may be used to denote only the rate of motion but not the ordering of the hydrocarbon chains.

The above mentioned techniques are not capable of providing a sufficiently detailed insight into membrane order and membrane fluidity. X-ray diffraction measures an average over the whole bilayer cross section and allows no conclusions about the fluctuations of the individual segments or about possible chain conformations. Also, the method is not sensitive enough to detect variations within the "fluid" state of two membranes of different chemical composition. ^{1}H and ^{13}C relaxation times, though characterized by a better resolution, do not easily yield to a quantitative interpretation. This is due to the fact that they depend in a complex fashion on the bilayer order as well as on the segmental rate of motion, both of which are unknown parameters. An approach which circumvents these difficulties is the use of ^{2}H NMR combined with selective deuteration of phospholipid molecules (Charvolin et al., 1973; Seelig and Niederberger, 1974; Seelig and Seelig, 1974; 1975; Stockton et al., 1975; 1976). In this case, the anisotropy of the motion and the extent of fluctuations can be inferred unambiguously from the quadrupole splitting of the deuterated segment. The first phospholipid bilayer to be studied by this method was dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) (Seelig and Seelig, 1974; 1975). We have now prepared selectively deuterated 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) with the deuterium label attached at 11 different positions of the palmitic acyl chain and also at the 9, 10 position of the oleic acyl chain. This lipid was chosen because it is probably one of the most common naturally occurring phospholipids (about 70% in egg yolk lecithin; Tattrie et al., 1968). Furthermore, since the corresponding ^{2}H NMR parameters are known for the fully saturated DPPC system, this facilitates the interpretation of the data obtained for the unsaturated bilayer. The purpose of this work then is to present a detailed quantitative comparison between the chain ordering in a saturated and an unsaturated bilayer membrane and to show how the influence of a local rigid element extends throughout the whole bilayer. In addition, we have obtained an approximate measure for the rates of motion in DPPC and POPC bilayers from EPR studies of intercalated stearic acid spin probes.

Materials and Methods

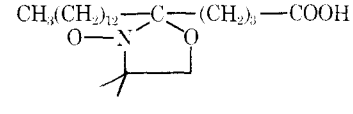
Synthesis of Selectively Deuterated 1-Palmitoyl-2-oleoyl-3-sn-phosphatidylcholine. Dipalmitoyl-3-*sn*-phosphatidylcholines (DPPC) selectively deuterated in both fatty acyl chains were synthesized as described earlier (Seelig and Seelig, 1974). DPPC was subjected to phospholipase A (from Serva, Germany) (*Crotalus atrox*) degradation. The lysophosphatidylcholine was reacylated with oleic acid anhydride according to a modified version of the procedure of Cubero Robles and van den Berg (1969). The acylation was carried out in the dark, under high vacuum at 40 °C. The reaction course was followed by thin-layer chromatography and was found to require 3–4 days for complete reacylation. Under these mild conditions, the acylation product is only slightly yellow, whereas at higher temperatures it turns brown. Intermigration of the fatty acyl chains is excluded at these low temperatures, as verified by deuterium magnetic resonance. The 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) was colorless after purification by column chromatography on silica gel 60 (Merck) using chloroform–methanol–water (65:25:4 v/v) followed by pure methanol as eluents. The purified POPCs were characterized by thin-layer chromatography, by ^{1}H and ^{2}H NMR, and by infrared spectroscopy.

Altogether, 12 different unsaturated phospholipids were prepared with the deuterium label attached at positions 2–6, 8–10, 12, 14, and 15 of the palmitic acyl chain and in the 9, 10 position of the oleic acyl chain. Oleic-9,10-d_2 acid was synthesized according to Khan (1952).

Preparation of the Bilayer Phase. The POPC in chloroform solution was filled in an ampule. The chloroform was evaporated first under nitrogen and then under high vacuum. Water (51.5 wt %) was added to the POPC (48.5 wt %) and the ampule was sealed under argon. A homogeneous phase was obtained by shaking the ampule at room temperature. All manipulations with POPC were performed in the dark.

Deuterium Magnetic Resonance Measurements. The ^{2}H NMR measurements were made at 41.4 and 13.8 MHz with a Bruker HX-270-FT and a HX-90-FT spectrometer, respectively, both equipped with a calibrated variable temperature unit.

Spin-Label EPR Measurements. As a spin-label, we have used stearic acid with the nitroxide group attached at carbon atom 5 (abbreviated as I (12.3)). A small amount of this label was intercalated into coarse dispersions of POPC and DPPC, containing about 20 mg of lipid/ml of water. EPR spectra were recorded on a Varian E-9 spectrometer.



I (12.3)

Results and Discussion

An example of a ^{2}H NMR spectrum of POPC bilayers in the liquid crystalline state at 42 °C is shown in Figure 1. The deuterium signals are rather sharp and the apparent line width

[1] Abbreviations used are: DPPC, dipalmitoyl-3-*sn*-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

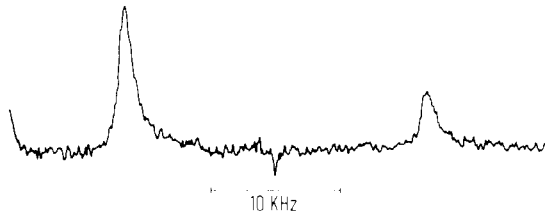


FIGURE 1: Deuterium magnetic resonance spectrum (41.4 MHz) of randomly oriented bilayers of 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine deuterated in the C-8 position of the palmitic acyl chain at 42 °C (48.5 wt % POPC; 51.5 wt % H_2O), 30 000 transients.

of the two peaks of the powder-type spectrum remains nearly constant upon lowering the temperature. However, below −5 °C the signal suddenly disappears. This temperature corresponds to the transition of the hydrocarbon chains from the disordered, liquid crystalline state to the ordered gel state and agrees with the transition temperature found by differential scanning calorimetry (T_c = −5 °C, de Kruyff et al., 1973).

Regarding the quantitative evaluation of deuterium resonance spectra, such as depicted in Figure 1, it should be noted that each deuteron of a CD_2 group contributes a doublet to the spectrum. In principle, the two deuteron splittings need not to have the same frequency separation; experimentally, however, only one quadrupole splitting is observed for all POPC bilayers deuterated in the palmitic acyl chain. This means that the two deuterium atoms per CD_2 group experience the same type of motion and are thus characterized by the same order parameters S_{CD} of the C—D bond vector. For a powder-type spectrum the order parameter S_{CD} is calculated from the residual quadrupole coupling $\Delta\nu_Q$ according to:

$$\Delta\nu_Q = (3/4)(e^2qQ/h)S_{CD} \quad (1)$$

The deuteron quadrupole splitting constant (e^2qQ/h) amounts to about 170 kHz for paraffinic C—D bonds (Burnett and Muller, 1971). Only the absolute value, $|S_{CD}|$, can be determined, since the sign of $\Delta\nu$ is unknown. Assuming axially symmetric rotation around an axis perpendicular to the plane spanned by the two C—D bond vectors, the segmental order parameter of this axis, S_{mol}, is related to that of the C—D bond vector by (Seelig and Seelig, 1974)

$$S_{mol} = -2S_{CD} \quad (2)$$

Ordering of the Palmitic Acyl Chain of POPC. The variation of the order parameters S_{mol} with temperature and with the position of the deuterium label in the chain is shown in Figure 2. To a first approximation, the *shapes* of the order profiles are independent of temperature, the effect of temperature being a shift of the whole curve up or down the ordinate. Decreasing the temperature induces a better ordering of the hydrocarbon chains. The order profiles in Figure 2 are characterized by a rather constant order parameter for the first 8 chain segments, followed by a gradual decrease of the chain order towards the methyl terminal. Considering just the *shape* of the curves, the order profiles of POPC bilayers resemble those established for bilayers composed of saturated phospholipids (Seelig and Seelig, 1974) or of soap-like molecules (Charvolin et al., 1973; Seelig and Niederberger, 1974).

A more detailed comparison, however, reveals two distinct differences between saturated and unsaturated phospholipid bilayers. This is illustrated in Figure 3 where the order parameters S_{mol} of DPPC and POPC bilayers are plotted (1) at the same temperature (here 42 °C, Figure 3A) and (2) at equal relative temperatures with respect to the corresponding gel-

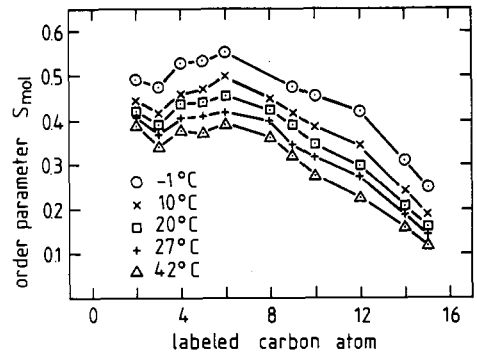


FIGURE 2: Order parameters S_{mol} as a function of the label position in the palmitic acyl chain of 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (48.5 wt % POPC; 51.5 wt % H_2O) at different temperatures.

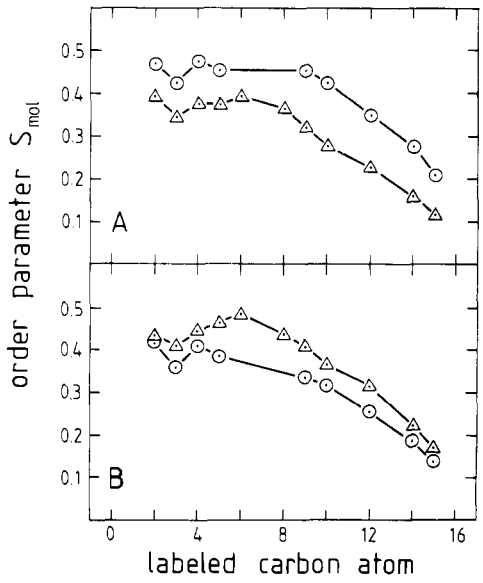


FIGURE 3: Order parameter S_{mol} as a function of the labeled carbon atom for (△) 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine and for (○) 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine, (A) At the same temperature (42 °C), (B) at equal temperatures relative to the respective phase transition temperatures; POPC at 14 °C (T_c + 19 °C); DPPC at 60 °C (T_c + 19 °C).

to-liquid crystal transition temperature (Figure 3B). If compared at the same temperature (Figure 3A), POPC bilayers are always less ordered than DPPC bilayers. The incorporation of a cis double bond obviously perturbs the parallel packing of the hydrocarbon chains, reducing in turn the ordering in *all* parts of the hydrophobic region. From the increase in the configurational freedom of the hydrocarbon chains, it immediately follows that the chains become also more contracted. The extent of contraction can be estimated from the order parameters, S_{mol}, which are related to the average length of the hydrocarbon chain, $\langle L \rangle$, in the bilayer according to (Seelig and Seelig, 1974):

$$\langle L \rangle = 1.25\left[n - 0.5\sum_i (1 - S_{mol}^{(i)})/1.125\right] \quad (3)$$

Here n is the number of C—C bonds per chain (effective length 1.25 Å) and $S_{mol}^{(i)}$ is the order parameter of the ith segment. The effective length, $\langle L \rangle$, of the palmitic acyl chain is, thus, found to be 12.8 Å in POPC and 13.7 Å in DPPC at 42 °C. The thickness of the complete POPC bilayer (containing two monolayers) is, thus, reduced by about 2 Å compared to the fully

saturated DPPC bilayer at the same temperature. Since it is well known from theories of the liquid-crystalline state that van der Waals forces are proportional to the average order in the system (Maier and Saupe, 1958; Marčelja, 1974), it can be concluded that these forces are smaller in POPC than in DPPC bilayers. A quantitative estimate using Marčelja's molecular field model (cf. Schindler and Seelig, 1975) indicates a decrease of the V_0 parameter by about 150 cal/mol. As a corollary of the reduced van der Waals forces, it follows from the theory that the gel-to-liquid crystal phase transition should occur at lower temperatures in POPC than in DPPC. This is in agreement with the experiment, since the transition temperature amounts to 41 °C for bilayers of DPPC and is lowered to −5 °C for POPC. The same calculation further shows that the number of gauche conformers per palmitic acyl chain increases from 4.2 in DPPC to 4.8 in POPC (at 42 °C).

It is also instructive to compare the two systems under conditions where they are in the same physical state, i.e., subjected to the same average molecular forces. According to theories describing phase transitions, such a situation exists at equal temperatures relative to the respective phase transitions. In Figure 3B, the order parameters of DPPC and POPC have, therefore, been plotted for temperatures 19 °C above the corresponding transition temperatures, illustrating the second effect associated with the double bond. The two order profiles can be seen to run parallel and closely together in the first part of the chain until carbon atom 4 and then again for the terminal region starting from carbon atom 10. This is in agreement with the theoretical expectation. In the intermediate chain region, the two curves depart, however, from each other, the divergence reaching its maximum around carbon atom 6. It should be noted that it is now the *unsaturated* system which is the more ordered one. This increase in the order parameter in POPC can be interpreted as a local stiffening effect caused by the cis double bond. The >C=C< segment itself is rigid and this restriction in the rotational freedom reduces the number of available rotational isomeric states in the adjacent palmitic acyl chain. The stiffening effect of the double bond obviously supersedes the increased rotational freedom around single bonds connected directly to the double bond.

Since the C=C bond in POPC is located between C-9 and C-10, it is somewhat surprising that the maximum difference between the order profiles of POPC and DPPC occurs not at the C-9 but at the C-6 segment of the palmitic acyl chain. This can be explained by assuming two different orientations for the beginnings of the palmitic and oleic acid residues with respect to the membrane surface. The physical inequivalence of the two fatty acyl chains has already been demonstrated for bilayers of DPPC in the liquid-crystalline state (Seelig and Seelig, 1975), as well as for crystals of dilauroylphosphatidylethanolamine (Hitchcock et al., 1974). In both systems, chain 1 (attached at glycerol C-1) is found to be oriented perpendicular to the bilayer, while chain 2 (attached at glycerol C-2) is found to be bent; i.e., the first segment of chain 2 is oriented parallel to the bilayer surface. Due to this bent conformation, all segments of chain 2 are shifted towards the bilayer surface and are positioned closer to the lipid–water interface than the corresponding segments of chain 1. Assuming a similar conformation for POPC bilayers, this implies that the double bond is nearer to the membrane surface than the C-9 and C-10 segments of the adjacent palmitic acyl chain. The stiffening caused by the C=C bond is then felt mainly by segments preceding the C-9 segment, which is in agreement with the experiment.

The importance of the attachment site for the physical properties of a lipid bilayer is also illustrated by thermodynamic measurements. The gel-to-liquid crystal transition temperatures of 1-stearoyl-2-oleoyl-3-*sn*-phosphatidylcholine and 1-oleoyl-2-stearoyl-3-*sn*-phosphatidylcholine are found to be 3 and 22 °C, respectively (Phillips et al., 1972; de Kruyff et al., 1972). Also, the transition enthalpies of the two systems differ by more than 30%. This provides some indirect evidence that the chain conformation in a bilayer depends not only on the chemical structure of the fatty acid but also on its position in the glycerol backbone. The transition temperature and the transition enthalpy of phospholipid bilayers are, furthermore, influenced by the location of the double bond with respect to the chain ends. Both parameters reach a minimum value for the cis double bond in the 9,10 position and increase almost up to the values for completely saturated phospholipids when the double bond is shifted towards either end of the chain (Barton and Gunstone, 1975).

Motion of the Double Bond. We now turn to a discussion of the motion of the double bond itself. The ^{2}H NMR spectrum of the POPC bilayers deuterated at the 9, 10 positions of the oleic acyl chains consists of *two* doublets with frequency spacings of 2.5 and 13.3 kHz, the order parameters $|S_{CD}|$ being 0.02 and 0.1, respectively (at 27 °C). This observation requires that the axis of motional averaging is not parallel to the double bond, since, if this was the case, the deuterons would be inclined at the same angle to the rotation axis and hence produce the same quadrupole splitting. Unfortunately, the available data are not sufficient for a quantitative description of the motion of the double bond as a whole. Assuming a C_{2v} symmetry for the cis ^{2}H—C=C—^{2}H fragment in the chain, it immediately follows that *three* independent order parameters are needed to completely specify the motion (cf. Saupe, 1964); these cannot be determined from only two experimental parameters.

The order parameters $|S_{CD}|$ of the olefinic deuterons are clearly smaller than those of the corresponding CD_2 deuterons in the adjacent chain. It would, however, be misleading to conclude that the oleic acyl chain is more disordered than the palmitic acyl chain. Order parameters are tensorial averages which depend not only on the statistical order of the system, but also on the orientation of the molecule-fixed tensor (here the electric field gradient tensor at the position of the deuterium nucleus) with respect to the axis of motional averaging, i.e., the normal to the bilayer surface. The low-order parameters of the olefinic deuterons are more likely to be caused by the chain bending due to the cis conformation than by an increase in disorder. This is suggested by spin-label studies of potassium oleate bilayers where *negative* spin-label order parameters were observed in the vicinity of the double bond (Axel and Seelig, 1973). This result indicates an ordered potassium oleate bilayer, which induces a bent conformation of the fatty acid spin probe in the region close to the cis double bond.

Comparison with Egg-Yolk Lecithin. The chain ordering in a related bilayer system, namely, egg-yolk lecithin, had been studied using both deuterated fatty acids (Stockton et al., 1976) and phospholipid spin-labels (Gaffney and McConnell, 1974) intercalated into the lipid membrane. Figure 4 shows a comparison of these investigations with our results for POPC. The shape of the curve drawn through the fatty acid deuterium order parameters (○) is similar to that of the pure POPC bilayer, although details of the order profile are smoothed out. The fatty acid order parameters are distinctly larger than those of the deuterated phospholipid (△). This discrepancy can be explained by the high percentage of deuterated fatty acid (~8 wt %) incorporated into egg-yolk lecithin in order to achieve

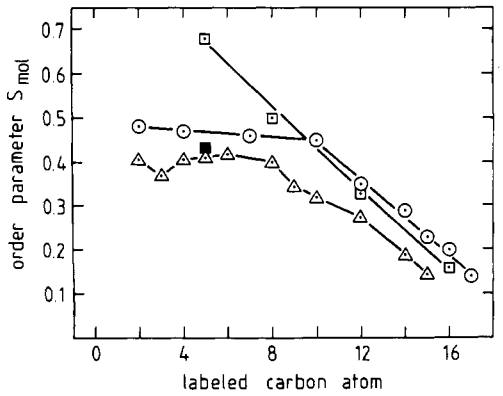


FIGURE 4: Order parameters as a function of the labeled carbon atom. (△) 1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine at 27 °C; (○) 20 mol % of deuterated stearic acid intercalated in egg lecithin lamellar dispersions at 30 °C (Stockton et al., 1976); (□) phospholipid spin-labels intercalated in egg lecithin at ~27 °C (Gaffney and McConnell, 1974); (■) spin-label result corrected for tilt effect.

reasonable signal-to-noise ratios. Incorporation of palmitic acid into a POPC bilayer deuterated at C-10 leads to an increase of the order parameter S_{mol} from 0.32 at 0 wt % palmitic acid to 0.44 at 15 wt % (at 27 °C). Appreciable amounts of free fatty acid, therefore, induce a better chain ordering. Spin-labeled fatty acids seem not to detect this difference. The use of deuterated fatty acids has, however, the advantage that it is readily applicable to the study of natural membranes where specific deuteration is difficult to achieve.

The spin-label order parameters require a more sophisticated explanation, since the discrepancy between spin-label EPR and ^2H NMR in this particular instance may arise from the different frequency range of the two methods. Spin-label EPR detects fast motions and the spin-label order parameter comprises fluctuations of frequencies $\gtrsim 10^8$ Hz. ^2H NMR has a lower frequency resolution and the deuterium order parameter measures fluctuations with frequencies $\gtrsim 10^5$ Hz. Motions with frequencies between 10^5 and 10^8 Hz are, therefore, *not* included in the spin-label order parameter but *are* included in the deuterium order parameter. If fluctuations of such intermediate frequencies exist in a lipid bilayer, the deuterium order parameter can be expected to be *smaller* than the spin-label order parameter. This is indeed the case in Figure 4. Gaffney and McConnell (1974) have interpreted their data in terms of a collective tilt of the hydrocarbon chains in the vicinity of the polar group (tilt angle ~30°). Assuming a lifetime of the tilt between 10^{-5} and 10^{-8} s, the same authors calculated a deuterium order parameter of 0.43 (■ in Figure 4) for the C-5 segment, in agreement with our experimental result. For a critical assessment of this finding, the following should, however, be noted. (1) Only for this particular bilayer are the deuterium data consistent with a *short-lived* tilting of the hydrocarbon chains. In two other bilayers, namely, a soap-like system and in DPPC, the deuterium order parameters are *larger* than the spin-label order parameters, excluding any time-scale difference and tilt effect (Seelig and Niederberger, 1974; Seelig and Seelig, 1974). (2) The synthetic POPC bilayer and the natural egg-yolk lecithin are not completely identical with respect to their chemical composition, their water content, and their gel-to-liquid crystal transition point. (3) Replacing the phospholipid spin-label by a fatty acid spin-label leads to a smaller spin-label order parameter (cf. Table I), which is now equal to the deuterium order parameter.

Rate of Motion. Let us briefly discuss the rate of motion in

TABLE I: Comparison of DPPC and POPC Bilayers Using the Stearic Acid Spin-Probe I (12.3).[a]

		DPPC	POPC
	$T_{\parallel}$ (Gauss)	22.25	20.6
	$T_{\perp}$ (Gauss)	9.75	10.7
Isotropic hyperfine splitting constant	a_N (Gauss)	14.5	14.5
Spin-label order parameter	S_3	0.46	0.36
Line width of central peak	ΔH (Gauss)	2.9	2.25

[a] Temperature 42 °C.

POPC bilayers. As an approximate experimental parameter, we have chosen the line width of the stearic spin probe I (12.3). When incorporated into POPC or DPPC dispersions, this label produces typical bilayer spectra with well-resolved inner and outer hyperfine extrema. The analysis of such spectra is straightforward (Seelig, 1970; Hubbell and McConnell, 1971) and the results are summarized in Table I. The quantity ΔH describes the line width of the central peak in the powder-type spectrum and is found to be smaller for POPC than for DPPC (at 42 °C). This suggests that the rate of reorientation of the nitroxide group is faster in POPC than in DPPC. Assuming that the motion of the spin-label is representative for the fluidity of the whole bilayer, this leads to the conclusion that the more disordered system is also the more fluid one. However, it is difficult to assess to which extent the changes in the line width reflect significant differences in intrinsic bilayer properties like the microviscosity or the lateral diffusion constant of the lipid molecules.

Summarizing the above information, it is evident that there are at least three different effects associated with the incorporation of a cis double bond into a bilayer membrane. First and most important, the double bond leads to a general decrease in the hydrocarbon chain ordering in all parts of the bilayer. Second, considering the *relative* flexibility of the saturated chain attached at position 1, this is found to be more restricted in the vicinity of the double bond than further away from it. Third, the increase in disorder seems to be accompanied by some increase in the rate of motion, at least as judged from the line shapes of spin-labels.

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Structural Basis of Heme Reactivity in Myoglobin and Leghemoglobin: Thermal Difference Spectra†

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ABSTRACT: Thermal perturbation difference spectra of sperm whale myoglobin (Mb) and soybean leghemoglobin a (Lb a) in the near-ultraviolet reveal similarities in the tryptophan environments of the two proteins. Of the two tryptophans in each protein, one has its indolyl NH group fully exposed to aqueous solvent, while the other behaves as if it were surrounded by motile but nonpolar residues with little access to water. These environments are not significantly altered by removal of the heme group. Assuming conformational homology, the helix-spacing role of Trp-A12 in Mb (Kendrew, J. C. (1962), *Brookhaven Symp. Biol. 15,* 216–228) may be taken over, in Lb a, by Trp-H8 which, though remote in linear sequence, would occupy a suitable spatial location. Thermal difference spectra in the Soret and visible regions of pure high-spin (fluoroferric) and pure low-spin (cyanoferric) complexes showed a red shift on cooling Mb complexes, reflecting a predominantly nonpolar environment around the heme, but a blue shift on cooling Lb complexes, reflecting a more solvent-exposed environment. Thermal difference spectra using rose bengal as a probe of the heme pockets in the two apoproteins supported these conclusions. Thermal difference spectra for the high-spin complexes of both Mb and Lb are slightly larger in magnitude than in the low-spin complexes. This may reflect a more flexible heme pocket in the high-spin state, as suggested by recent circular dichroic results. A structural basis for the high oxygen affinity of Lb compared with Mb is proposed, based upon the observed differences in polarity and flexibility of the heme pocket and in amino acid substitutions.

Mammalian myoglobins and plant leghemoglobins are the contemporary products of an evolutionary divergence occurring some 1200 million years ago. The observation of homologies in sequence (e.g., Dayhoff, 1972) and folding (Vainshtein et al, 1975) is all the more remarkable. The work described here forms part of studies designed to compare the structures of mammalian and plant hemoglobins with respect to folding, heme, and chromophore environment and their relationship to oxygen and ligand-binding effects. The technique of thermal perturbation difference spectroscopy is applied.

Thermal perturbation difference spectroscopy of proteins was developed by three groups (Bello, 1969a; Cane, 1969; Smith, 1970; Leach and Smith, 1972). In principle, it is analogous to the solvent perturbation difference spectral method introduced by Herskovits and Laskowski (1960), except that the perturbant employed is a small change in temperature rather than a change in solvent. Both techniques have been used to determine the degree of exposure of aromatic amino acids (mainly tyrosine and tryptophan) in native proteins. There are some advantages of thermal over solvent perturbation for such purposes. (1) The perturbation (temperature difference) can be varied continuously on the same sample. (2) If the sample temperature is cooled by say 10 °C, such a perturbation is less likely to cause conformational changes in the protein than is a change from aqueous solvent to 20–40% organic solvent. (3) The baseline can be internally checked by recording a difference spectrum when both sample and refer-

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