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# Orientation and Motion of Amphiphilic Spin Labels in Hexagonal Lipid Phases

**(membrane/cardiolipin/gangliosides/$Ca^{++}$/phase transition)**

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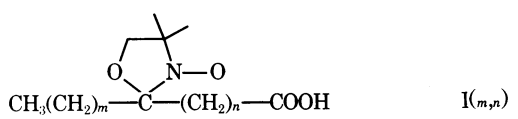
**ABSTRACT The acyl chain of spin-labeled fatty acids intercalates between the lipid hydrocarbon chains in hexagonal and micellar phases with the carboxyl group anchored at the lipid water interface. The spectra are characteristic of anisotropic motion and cannot be distinguished from the spectra of these probes in lamellar dispersions. In the hexagonal and micellar phases the molecular motion of the spin label increases as it is moved further away from the carboxyl group, similar to the behavior in the lamellar phase (Jost *et al.* (1971) *J. Mol. Biol.* 59, 77–98). The similarity in packing of the acyl chains in the hexagonal and lamellar phases suggests that localized regions of hexagonal phase are compatible with a bilayer matrix.**

It is well known that lipids can exist in various structures dependent on conditions such as water content, temperature, presence of cations, etc. (1). This polymorphic behavior may be maintained in biological membranes. Physical studies of biological membranes show that the predominant membrane structure is lamellar. The major membrane phospholipids have been shown by various physical techniques to exist in a bilayer configuration in aqueous dispersions (21), and this system is often used as a model for biological membranes. However, some lipids in localized regions may be capable of reversible phase transitions, due to changes in the environment of the membrane, for specialized structural and functional roles not shared by other common membrane lipids.

Electron spin resonance (ESR) spin labeling is now widely used to study the structural organization of model and biological membranes. Amphiphilic fatty acid (2, 4) or phospholipid spin labels (5) orient in a lamellar structure such that the preferred orientation of the long axis is perpendicular to the membrane surface, and gives spectra characteristic of anisotropic motion about the long axis. In our recent investigation of the structural organization of the major classes of membrane lipids (10), we found that 5-doxyl palmitic acid had anisotropic motion in known hexagonal phases similar to that in the lamellar phase of lipids.

In this paper we have selected three lipid systems to explore the orientation and motion of fatty acid spin labels I($m$,$n$) in hexagonal and micellar phases.




I($m$,$n$)

Comparison between hexagonal and lamellar phases was possible for the first two systems. These stystems are (*i*) cardiolipin-$Na^+$ (lamellar phase) and cardiolipin-$Ca^{+2}$ (hexagonal cylindrical phase) (8, 9) (*ii*) 1-monoglycerides in semi-hydrated planar films (lamellar) and fully-hydrated (hexagonal), and (*iii*) gangliosides (micellar) (12, 13). From these studies we show (*a*) fatty acid spin labels I($m$,$n$) have preferred orientation in cylindrical and spherical micellar phases with the long axis parallel to the lipid acyl chains, resulting in spectra characteristic of rapid anisotropic motion and (*b*) there is a fluidity gradient in the hexagonal phase, i.e., the motional freedom of the acyl chains increases toward the terminal methyl group as has been observed for the lamellar phase (2, 4, 5).

## MATERIALS AND METHODS

5-Doxyl-palmitic acid [I(*10,3*)] and 8-doxyl-palmitic acid [I(*7,6*)] were prepared according to the method of Hubbell and McConnell (5), and 12-doxyl-stearic acid [I(*5,10*)] was prepared according to the method of Keana *et al.* (18). Cardiolipin from beef heart, monoglycerides from pig liver, and gangliosides from bovine brain were purchased from Serdary Research Laboratory, London, Ontario. The fatty acid composition of these lipids has been reported (10). Egg lecithin was purchased from Pierce Chemical Co. Cholesterol was recrystallized from methanol (melting point, 148.5°).

Liposomes were prepared by hand dispersion of a dry film of lipid (about 1 mg) and spin label in 0.05 ml of 20 mM phosphate buffer containing 0.15 M NaCl at pH 7.2. The $Ca^{+2}$–cardiolipin complex was prepared by dispersing the lipid in 75 mM $CaCl_2$ at pH 7.2. The heavy waxy precipitate was mounted in a standard ESR tissue cell or was squeezed between two flat quartz plates for ESR measurements. Planar lipid films were prepared and hydrated as described (6, 7). The lipid to spin-label ratio in both films and dispersions was 150:1. All spectra were recorded at room temperature, or at 44° for gangliosides, on a Varian E-6 ESR spectrometer. The temperature was regulated by a Varian variable-temperature controller.

All samples were examined under a polarizing microscope with a heating stage, both in dispersions and films. For films a small amount of the lipid in chloroform was dried on a glass cover slip under a stream of $N_2$, hydrated with buffer solution, and sealed with a cover slip. The isotropic film could be disrupted to show birefringence by pushing on the cover slip.

## RESULTS

### Orientation and motion of fatty acid spin label in the hexagonal phase of cardiolipin

Calcium converts the lamellar phase of cardiolipin in water to a precipitate that is a pure hexagonal phase of type $H_{II}$ with water cylinders in a hydrocarbon matrix, as depicted in Fig. 1*B* (8, 9). This precipitate labeled with 5-doxyl-palmitate and 8-doxyl-palmitate gives spectra characteristic of anisotropic motion of the probe about its long axis. The precipitate mounted in a flat tissue cell showed some orientation dependence, indicating some degree of orientation of the cylinders with respect to the plane of the tissue cell. The pre-

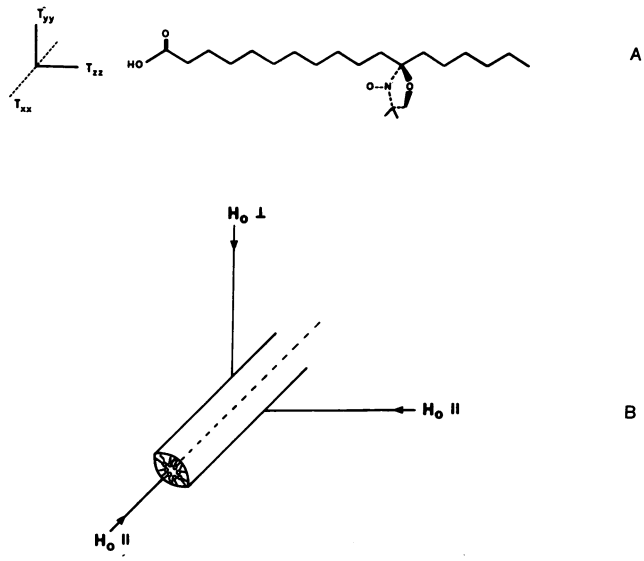



FIG. 1. (A) Fatty acid spin label and orientation of hyperfine tensor components. (B) Diagram of hexagonal II cylinder with lipid chains on the outside, water on the inside. When cylinders are sheared between two plates the magnetic field applied perpendicular to the plate ($H_0 \perp$) is perpendicular to the long axis of the cylinder. The magnetic field applied parallel to the plate ($H_0||$) is perpendicular to some cylinders and parallel to others.

cipitate was then squeezed between two quartz plates in order to improve the orientation of the long axes of the cylinders parallel to the plate. The orientation dependence of the spectra with the magnetic field applied perpendicular and parallel to the plane of the plate is shown in Fig. 2. For these spin labels the maximum component of the hyperfine tensor, $T_{zz}$, is approximately parallel to the long axis of the extended hydrocarbon chain, as indicated in Fig. 1. Therefore, the outer hyperfine extrema arise from probe molecules parallel to the applied magnetic field $H_0$ ($H_0||T_{zz}$), while the inner hyperfine extrema arise from probe molecules perpendicular to $H_0$ ($H_0||T_{xx}$ and $T_{yy}$). The magnetic field applied parallel to the plate is parallel to some cylinder axes and perpendicular to others. When the plate is oriented perpendicular to the magnetic field, most of the cylinder axes are perpendicular to $H_0$. The increase in relative intensity of the outer extrema in the perpendicular orientation indicates that the preferred orientation of the long axis of the probe is perpendicular, rather than parallel, to the cylinder axis. Thus, the doxyl fatty acids must be oriented in the hexagonal phase intercalated between the hydrocarbon chains of the lipids, with the carboxyl group anchored at the polar head group.

**Lamellar and hexagonal phases of 1-monoglycerides**

ESR spectra of the fatty acid probes in oriented films of monoglycerides are orientation dependent (Fig. 3*B*) when the film is hydrated under 100% relative humidity, indicating multibilayer structure. Heating the film to about 80° and allowing it to cool down again before hydration was necessary to obtain orientation of the probe. X-ray diffraction studies show that 1-monoglyceride containing dodecanoic acid is lamellar at 44° at low water concentrations (26). However, when the film is fully hydrated the spectra are no longer orientation dependent, but are characteristic of anisotropic motion (Fig. 3*A*). In fact, they are almost exactly like the

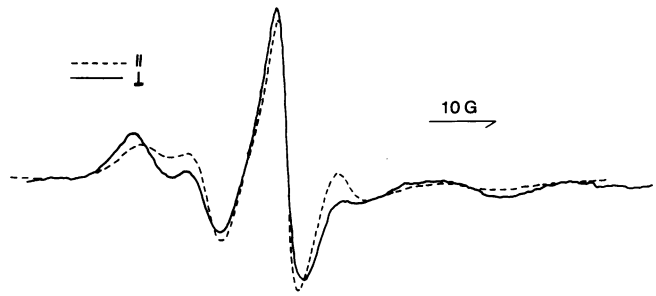



FIG. 2. ESR spectra of 5-doxyl-palmitate in hexagonal-phase $Ca^{+2}$-cardiolipin squeezed between two plates with the magnetic field applied perpendicular and parallel to the plane of the plate.

spectra of dispersions (Fig. 4 and Table 1). Washing of the film with buffer resulted in no noticeable loss of lipid and no significant loss of spin-label signal. Thus, the lipid is not dispersed when fully hydrated and the anisotropic motion must arise from a hexagonal or micellar phase of type II. A dispersion of this preparation (fatty acid content 22% *16:0*, 56% *18:0*, 17% *18:1*) consisted of large white particles suspended in a clear solution that were not birefringent under a polarizing microscope. An isotropic film of the lipid, when disrupted under the polarizing microscope, showed the birefringent fans characteristic of the hexagonal cylindrical phase (11). However, the orientation independence of the spectra of the planar film suggests a spherical micellar phase with the hydrocarbon chains on the outside, since it is probable that cylinders would line up on the flat faces of the quartz cell leading to orientation dependence as in Fig. 2.

**Micellar phase of gangliosides**

Gangliosides are known to exist in micellar solution with the polar head groups in the aqueous phase and the hydrocarbon chains toward the inside (12, 13). Dispersions of gangliosides form a clear solution, while in the presence of an equimolar amount of cholesterol they form a milky solution. The phase in the presence of cholesterol is not known but dispersions in the presence and absence of cholesterol are not birefringent, indicating spherical micelles in both cases. At room tem-

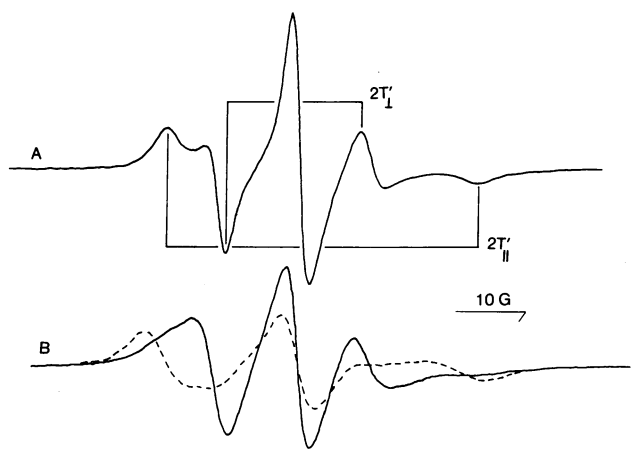



FIG. 3. ESR spectra of 8-doxyl-palmitate in a planar film of 1-monoglycerides, (*A*) in the fully hydrated state—both orientations are identical, only one is shown, and (*B*) in the semi-hydrated state, with the applied magnetic field perpendicular (——) and parallel (– – –) to the long axis of the spin label.

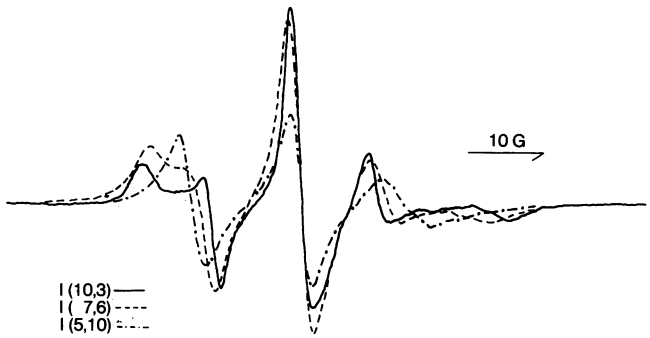



FIG. 4. ESR spectra of 5-doxyl-palmitate (——), 8-doxyl-palmitate (– – –), and 12-doxyl stearate (–·–·–) in dispersions of 1-monoglycerides (hexagonal phase, pH 7.2).

perature the probe is relatively immobilized, but at 44° it gives spectra characteristic of rapid anisotropic motion in the presence and absence of cholesterol (Table 1).

**Fluidity gradient in the hexagonal phase**

The degree of anisotropy of the spectra can be measured as $\Delta T = T'_{||} - T'_{\perp}$, where $T'_{||}$ and $T'_{\perp}$ are the hyperfine splittings as indicated in Fig. 3 (5). This property is a measure of the disorder of the probe. By use of a series of fatty acid spin labels with the nitroxide group located at different positions, the motional freedom along the chain can be studied (2, 4, 5). Table 1 shows that there is a decrease in $\Delta T$ as the nitroxide group moves away from the polar head group in all of the hexagonal phases, similar to that in the lamellar phases (Table 2). Indeed 12-doxyl stearate is nearly isotropic in all cases. This finding indicates the presence of a fluidity gradient in the hexagonal phase similar to that which has been observed in the lamellar phase (2, 4, 5).

TABLE 1. *Spectral parameters of doxyl fatty acids in some hexagonal or micellar lipid phases*

| Spin label* | Lipid system | $T'_{\perp}$ | $T'_{||}$† | $\Delta T$ |
|---|---|---|---|---|
| I(*10,3*) | $Ca^{+2}$–Cardiolipin | 9.0 | 25.2 | 16.2 |
| I(*7,6*) | | 9.5 | 24.7 | 15.2 |
| I(*5,10*) | | 13.9 | | — |
| I(*10,3*) | 1-Monoglycerides | 9.9 | 24.3 | 14.4 |
| I(*7,6*) | dispersion | 10.3 | 22.6 | 12.3 |
| I(*5,10*) | | 14.6 | | — |
| I(*10,3*) | 1-Monoglycerides | 9.9 | 24.2 | 14.4 |
| I(*7,6*) | planar film-fully | 10.1 | 23.6 | 13.5 |
| I(*5,10*) | hydrated‡ | 14.3 | | — |
| I(*10,3*) | Gangliosides dis- | 10.6 | 23.0 | 12.4 |
| I(*7,6*) | persion, 44° | 14.7 | | — |
| I(*5,10*) | | 14.9 | | — |
| I(*10,3*) | Gangliosides– | 9.5 | 25.9 | 16.4 |
| I(*7,6*) | cholesterol 1:1 | 9.1 | 27.4 | 18.3 |
| I(*5,10*) | dispersion, 44° | 14.5 | | — |

* I(*10,3*) is 5-doxyl-palmitate, I(*7,6*) is 8-doxyl-palmitate, and I(*5,10*) is 12 doxyl-stearate.

† $T'_{\perp}$ and $T'_{||}$ measured as shown in Fig. 3.

‡ $T'_{\perp}$ and $T'_{||}$ for planar film measured in one orientation of film.

It is interesting to compare the fluidity of the hexagonal and lamellar phases more closely. Comparison of $\Delta T$ for the hexagonal $Ca^{+2}$–cardiolipin (Table 1) with $\Delta T$ for the lamellar phases of dispersions of $Na^{+}$–cardiolipin (8) and egg lecithin (Table 2) indicates that the hexagonal phase is not as fluid near the polar head-group region as the lamellar phase. For 5-doxyl-palmitate in dispersions of monoglycerides (hexagonal), $\Delta T$ is larger than for partially hydrated oriented films of monoglycerides (lamellar). However, it is difficult to compare quantitatively the $\Delta T$ values between partially hydrated oriented multibilayers and dispersions for two reasons. First, the water content differs. But, the partially hydrated lipid would be expected to be less fluid than when fully hydrated in dispersions (ref. 4 and unpublished data), as is true for the lamellar phase of monoglycerides containing an equimolar amount of cholesterol (Table 2). Second, measurements of the hyperfine splitting constants in dispersions do not correspond exactly with those in oriented films, even if the water content and phase are identical (19). However, since the $\Delta T$ values for 8-doxyl palmitate in the hexagonal dispersions and the lamellar film are identical, the larger $\Delta T$ value for 5-doxyl palmitate in the hexagonal phase is consistent with the concept of tighter packing in the polar head-group region of the hexagonal phase than in the lamellar phase, while the region near the ends of the chains is quite fluid.

A consideration of the fluidity of gangliosides (Table 1) shows that cholesterol has the same effect in decreasing the fluidity of the lipid chains in this micellar phase as has been observed (5–7) for the lamellar phase.

## DISCUSSION

In this paper, we have shown that the use of fatty acid spin labels cannot distinguish between the hexagonal phase and an isotropic distribution of vesicles with lamellar structure. In order to demonstrate the existence of a substantial amount of

TABLE 2. *Spectral parameters of doxyl fatty acids in some lamellar lipid phases*

| Spin label | Lipid system | $T'_{\perp}$ | $T'_{||}$* | $\Delta T$ |
|---|---|---|---|---|
| I(*10,3*) | $Na^{+}$–cardiolipin disper- | 9.4 | 25.3 | 15.9 |
| I(*7,6*) | sion | 9.9 | 23.4 | 13.5 |
| I(*5,10*) | | 14.3 | | — |
| I(*10,3*) | Egg-lecithin dispersion | 9.1 | 25.4 | 16.3 |
| I(*7,6*) | | 9.9 | 23.6 | 13.7 |
| I(*5,10*) | | 14.1 | | — |
| I(*10,3*) | 1-Monoglycerides planar | 9.4 | 22.1 | 12.7 |
| I(*7,6*) | film-partially hydrated | 9.4 | 21.7 | 12.3 |
| I(*5,10*) | | 13.9 | 15.4 | 1.5 |
| I(*10,3*) | 1-Monoglyceride–choles- | 7.9 | 26.1 | 18.2 |
| I(*7,6*) | terol 1:1, planar film | 7.7 | 26.1 | 18.4 |
| I(*5,10*) | partially hydrated | 8.4 | 22.5 | 14.1 |
| I(*10,3*) | 1-Monoglyceride–choles- | 8.9 | 25.8 | 16.9 |
| I(*7,6*) | terol 1:1, planar film | 8.9 | 26.7 | 17.8 |
| I(*5,10*) | fully hydrated | 8.9 | 24.0 | 15.1 |

* For dispersions $T'_{||}$ and $T'_{\perp}$ measured as shown in Fig. 3. For planar films $T'_{\perp}$ was measured as for dispersions to facilitate comparison of hexagonal and lamellar phases, but $T'_{||}$ was measured as the separation of the midpoints of the low- and high-field peaks.

lamellar structure in biological membranes or model systems by spin labeling, the membrane surfaces must be oriented in some way—as by shearing (2) or flattening by centrifugation (16)—unless the spin label used can be shown to differentiate between the lamellar and other phases. In a recent paper from our laboratory in which lamellar structure was detected in the inner membrane of mitochondria by the use of a spin-labeled 2,4-dinitrophenol, this spin label had no anisotropic motion in $Ca^{+2}$–cardiolipin or mono- and di-glycerides (micelles) (28). Model membrane systems can be studied as planar-oriented films (3, 6, 17), and offer the best method for eliminating the contribution of an isotropic distribution of membrane surfaces to the resonance anisotropy.

Although biological membranes probably contain a large percentage of lamellar structure, as indicated by x-ray diffraction (14) and electron microscopy (15), they may also contain localized regions of hexagonal or other complex structures for specialized functions. The broad thermal transitions observed by differential scanning calorimetry in mixtures of lipids have been attributed to clustering of lipids with similar hydrocarbon chains when the temperature is not above that of the transition range (23). If similar clustering of lipids with different polar head-groups occurs, even small concentrations of certain specialized lipids could have an important effect on cell membrane function. Some evidence has been presented for clustering of lipids with similar hydrocarbon chains in the membrane of *Escherichia coli*, allowing optimal activity of proline uptake and succinate dehydrogenase (27).

The relative concentrations of these specialized lipids may lead to cell membrane specialization. Cardiolipin exists in high concentration (21.5%) in the inner mitochondrial membrane, as compared to 0.5% in endoplasmic reticulum (22). Gangliosides exist in highest concentration in synaptosomal membranes (23). Excessive concentrations of this lipid are found in Tay–Sachs disease. High molar concentrations of gangliosides convert phosphatidylcholine from the lamellar to the micellar phase (13).

It has been pointed out that if the hexagonal structure is stabilized even transiently in the plane of a lamellar membrane—for example, by changes in $Ca^{+2}$ ion available for interaction with cardiolipin—the permeability properties of the membrane would be greatly changed (24, 8). The hexagonal phase has been suggested to be involved in the reversible fusions induced by $Mg^{+2}$ ion in the mitochondrial inner membranes (25). The similarity in fluidity of the lipids in the hexagonal and lamellar phases that we have detected indicates similar binding forces between the acyl chains in both phases. Thus, the energy of transition from one phase to the other is probably low, and localized phase transitions might occur reversibly upon slight perturbations.

Localized regions of certain lipids may also be important for lipid–protein interactions and protein function. X-ray diffraction studies of protein–lipid model systems have revealed complex, highly-ordered structures in addition to lamellar phases (20).

In order to understand the functional roles of these lipid phase transitions it is necessary to study the structural properties of these complex phases and to investigate the conditions that can induce phase transitions in a mixture of lipids. In our opinion, these complex structures cannot be detected in biological membranes by ESR spin labeling, due to the impossibility of obtaining good orientation of the sample. However, it may be possible to use planar model membrane systems to detect phase transitions due to ions, drugs, and lipid–protein interactions.

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