The Dynamic Structure of Lipid Membranes. A ^{13}C Nuclear Magnetic Resonance Study Using Spin Labels†

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ABSTRACT: Biological membranes possess major regions of lipid bilayer. To study the dynamic structure of lipid bilayers, the ^{13}C nuclear spin–lattice (T_1) relaxation times of sonicated aqueous dispersions of egg yolk phosphatidylcholine (EYL) were measured. It was found that the T_1 values increase away from the glycerol backbone both toward the trimethylammonium group of the choline moiety and toward the terminal methyl group of the fatty acyl chains, thus indicating the presence of two mobility gradients. Information concerning the positioning of the stearic acid derivative spin labels has been obtained from T_1 measurements of phosphatidylcholine vesicles labeled with these probes. The results suggest that the carboxylic acid group of the fatty acid spin label is positioned in the vicinity of the phosphate groups of the phosphatidylcholine bilayer. The effect on T_1 due to the presence of spin label suggests that the fatty acyl chains of both EYL and the spin label swing up toward the glycerol backbone with the amplitude of the motion being larger for EYL. Thus, a "whipping" motion of the fatty acyl chains occurs as the lipids diffuse laterally in the plane of the membrane. The data also suggest that the trimethylammonium group of the choline is relatively mobile and spends some time in the vicinity of the phospholipid glycerol backbone.

A variety of approaches including X-ray diffraction (Engelman, 1970, 1971; Wilkins *et al.*, 1971), differential scanning calorimetry (Steim *et al.*, 1969), and spin-label electron spin resonance (esr) (Hubbell and McConnell, 1969) have indicated that biological membranes contain major regions of lipid bilayer. To investigate the details of the dynamic organization of lipid bilayers, many workers have studied sonicated aqueous lipid dispersions using spin-label esr and nuclear magnetic resonance (nmr). Spin-label esr studies of lecithin dispersions have demonstrated the existence of a mobility gradient in lipid bilayers, *i.e.*, there is an increase of motion in the fatty acyl chain as the terminal methyl group is approached (Hubbell and McConnell, 1971; Jost *et al.*, 1971). A mobility gradient has also been observed in ^1H nmr studies of sonicated egg yolk phosphatidylcholine (EYL)[1] dispersions (Horwitz *et al.*, 1972, 1973; Kohler *et al.*, 1972; Lee *et al.*, 1972), in natural abundance ^{13}C nmr studies of dipalmitoylphosphatidylcholine and dioleyllecithin dispersions (Metcalfe *et al.*, 1971; Levine *et al.*, 1972a; Metcalfe, 1972), and in ^{19}F nmr studies of monofluorostearic acids incorporated into EYL vesicles (Birdsall *et al.*, 1971a). These mobility gradients in lipid bilayers have been demonstrated to exist in a variety of biological membranes including those of *Mycoplasma laidlawii* (Rottem *et al.*, 1970), erythrocytes (Hubbell and McConnell, 1969; Landsberger *et al.*, 1971b), mitochondria (Keith *et al.*, 1970; Williams *et al.*, 1972), sarcoplasmic reticulum (Seelig and Hasselbach, 1971), influenza virus (Landsberger *et al.*, 1971a), Rauscher murine leukemia virus (Landsberger *et al.*, 1972), and SV5 virus (Landsberger *et al.*, 1973).

The rapid lateral diffusional translation of lipids and other membrane components parallel to the plane of the membrane has been demonstrated by a number of workers (Frye and Edidin, 1970; Kornberg and McConnell, 1971a; Nicolson *et al.*, 1971a,b; Overath *et al.*, 1971; Devaux and McConnell, 1972; Sackmann and Träuble, 1972; Scandella *et al.*, 1972; Devaux *et al.*, 1973; Lee *et al.*, 1973).

In the study of lipid membranes, natural abundance Fourier transform (FT) ^{13}C nmr (Oldfield and Chapman, 1971; Metcalfe *et al.*, 1971; Batchelor *et al.*, 1972; Levine *et al.*, 1972a; Metcalfe, 1972; Keough *et al.*, 1973) offers considerable resolution enhancement over conventional ^1H nmr (Horwitz *et al.*, 1972, 1973; Kohler *et al.*, 1972; Lee *et al.*, 1972). The increased resolution of ^{13}C nmr spectroscopy, however, is offset by the low abundance (1.1%) of naturally occurring ^{13}C nuclei.

To investigate the details of the motion of the fatty acyl chains of both EYL and the stearic acid derivative spin labels in lecithin membranes, the effect of these spin labels on the ^{13}C nuclear spin–lattice relaxation times of EYL has been measured. The data provide information on (1) the vertical positioning of the nitroxide spin label in a lipid bilayer, *i.e.*, the carboxylic acid group of the spin label appears to be located near the phosphate groups of the EYL bilayer; (2) the difference in the amplitude of the "whipping" motion of the fatty acyl chains of EYL and the stearic acid derivative labels, *i.e.*, the motional amplitude of the EYL fatty acyl chains is larger than that of the spin label hydrocarbon chain; and (3) the trimethylammonium group of the choline moiety is mobile and spends some time in the vicinity of the glycerol backbone of EYL.

Materials and Methods

Chemicals. Egg yolk phosphatidylcholine was isolated from fresh egg yolk according to the procedure of Singleton *et al.* (1965) with only minor modifications. All solvents and buffers were thoroughly deoxygenated. Only a single spot was observed in thin-layer chromatography of the product on silica gel plates (J. T. Baker Chemical Co., Phillipsburg, N. J.) de-

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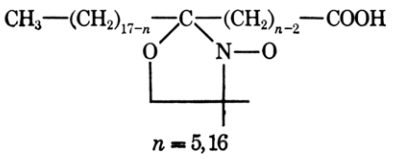
[1] Abbreviations used are: EYL, egg yolk phosphatidylcholine; FT, Fourier transform; PRFT, partially relaxed Fourier transform; CPK, Corey, Pauling, and Koltun.

veloped with chloroform–methanol–water (65:25:4). The purified egg lecithin was lyophilized from benzene and stored under nitrogen at −20°. (No benzene ^{13}C nmr resonance was detected.) The stearic acid derivative spin labels C_n were ob-



tained from Syva, Palo Alto, Calif., and were used without further purification.

Preparation of Spin-Labeled Egg Lecithin Liposomes. Egg lecithin samples for ^{13}C nmr were prepared by dissolving weighed amounts of lecithin and spin label in chloroform. The lecithin:spin label molar ratio was 60:1 for all experiments. Removal of the chloroform under reduced pressure followed by extensive pumping on a vacuum line produced a dry thin lipid film. (No chloroform ^{13}C nmr resonance was observed.) A slurry was made using 0.1 M KCl–5 mM Tris buffer (pH 8.0) (Sears, 1972) by agitation on a vortex mixer. The concentration of lecithin usually was 0.2 g/ml. The slurry was sonicated for approximately 1 hr at power level 3 under a nitrogen atmosphere using a Sonifier cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) equipped with a microtip. During the sonication procedure the sample tube was immersed in an ice water–salt bath holding the temperature of the sample below 25°. After sonication no degradation of the EYL was detected by thin-layer chromatography. The sample was centrifuged at 4° for 30 min at 40,000 rpm using a Beckmann L3-50 ultracentrifuge with a SW50 rotor to remove undispersed lipid (Huang, 1969), and then transferred under nitrogen to an nmr tube. To prevent air from entering the sample tube, it was covered with a plastic cap using vacuum grease as a sealer.

Nmr Measurements. All ^{13}C nmr spectra were obtained at approximately 34° on a home-built nmr spectrometer operating at 15.08 MHz utilizing spinning 13-mm sample tubes (Wilmad Glass Co., Buena, N. J.) as previously described by Allerhand *et al.* (1971a). The apparatus includes a 14.1-kG magnet, a Fabri-Tek 1074 signal averager, a PDP-8/I computer, external ^{19}F field-frequency lock, and noise-modulated proton decoupling. The normal Fourier transform (FT) nmr spectra were obtained by the Fourier transform of the accumulated free induction decay following a $\pi/2$ pulse yielding a spectrum equivalent to the continuous-wave spectrum. The partially relaxed Fourier transform (PRFT) spectra were obtained employing a π–τ–$\pi/2$ pulse sequence, where τ is the delay time between the π and $\pi/2$ pulses (Vold *et al.*, 1968; Allerhand *et al.*, 1971b). The pulse sequence was repeated after a delay time at least four times greater than the largest T_1 value to be measured. The usual procedure was to obtain 2048 accumulations and to perform a 4K Fourier transform.

Esr Measurements. All esr spectra were recorded on a Varian E-4 spectrometer.

Electron Microscopy. The electron microscopy procedure as described by Sheetz and Chan (1972) was used with only minor modification. A drop of diluted egg lecithin vesicle solution (4–8 mg/ml of lecithin) was applied to a 300-mesh copper grid coated with Formvar. After 30 sec, the liquid was drawn off with a blotter and a drop of 2% phosphotungstic acid was applied. Thirty seconds later the grid was blotted and allowed to dry. The grids were observed with a Hitachi HU11A electron microscope operating at 50 kV. Polystyrene latex beads of diameter 880 ± 80 Å (Dow Chemical Co., Midland, Mich.) were used for calibration of the electron micrographs.

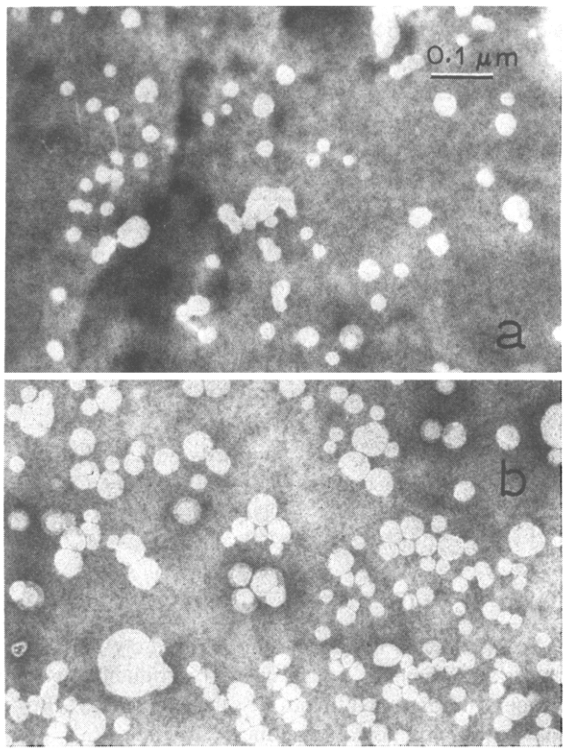


FIGURE 1: Electron micrographs of sonicated aqueous dispersions of egg yolk phosphatidylcholine. (a) Vesicle dispersion labeled with C_5 spin label 1 hr after sonication and (b) unlabeled vesicle dispersion 3 days after sonication. No differences were observed between labeled and unlabeled preparations.

Results

Electron micrographs of sonicated dispersions of EYL (*cf.* Figure 1) indicate the presence of a distribution of vesicle sizes (200–1200 Å in diameter), in agreement with Huang's (1969) observations, whereas unsonicated dispersions exhibit a range of vesicle sizes (250 to >4000 Å in diameter). The small vesicles (approximately 250 Å) vastly predominate in the sonicated dispersions, whereas a great number of very large aggregates with diameters greater than 4000 Å are observable in unsonicated dispersions. It has been shown that enhanced resolution in the nmr spectrum is obtained with the small lipid vesicles obtained by prolonged sonication of an aqueous dispersion of lipids (Chan *et al.*, 1971, 1972; Finer *et al.*, 1972a,b; Sheetz and Chan, 1972). To determine the effect of lengthy relaxation measurements on vesicle size, an electron micrograph was obtained of a 3-day old sample (*cf.* Figure 1b). Although some vesicle fusion was observed, resulting in a few large particles, most of the vesicles remained small. Since the total amount of time required for a set of PRFT spectra is 24–60 hr, some vesicle fusion probably occurs during the course of an experiment. Since no major differences were noted between the normal Fourier transform spectra obtained at the beginning and the end of a PRFT experiment, it is not likely that the results reported here are strongly influenced by changes in vesicle size during an experiment. No detectable differences were noted by electron microscopy between spin-labeled and unlabeled EYL dispersions.

TABLE I: Spin–Lattice Relaxation Times for Unlabeled Dispersions ($(T_1)_{lecithin}$) and Dispersions Labeled with the C_5 and C_{16} Spin Labels ($(T_1)_{Cn}$).[a]

Peak[b] No.	Chemical Shift[c]	Assignment[d]	$(T_1)_{lecithin}$ (sec)	$(T_1)_{C_5}$ (sec)	$(T_1)_{C_{16}}$ (sec)	$(1/T_{1N})_5$ (sec^{-1})	$(1/T_{1N})_{16}$ (sec^{-1})
1	19.2	Carbonyl	1.8	0.38	1.0	2.1	0.41
2	63.1	$-CH=\underline{CH}-CH_2-\underline{CH_2}-$	0.57	0.34	0.51	1.2	0.21
3	64.9	$-CH=\underline{CH}-CH_2-\underline{CH}=CH-$	0.75	0.39	0.60	1.2	0.33
4	126.5	Choline $-\underline{CH_2}N-$	0.30				
5	130.3	Glycerol $-\underline{CH_2}-$					
6	133.1	Choline $-O-\underline{CH_2}-$	0.41				
7	138.7	Choline $-N-(\underline{CH_3})_3$	0.62	0.37	0.54	1.1	0.24
8	158.7	$-\underline{CH_2}CO_2-$	0.26				
9	160.8	$-\underline{CH_2}CH_2CH_3$	0.64	0.48	0.37	0.52	1.1
10	162.8[e]	Main fatty acyl $(\underline{CH_2})_n$	0.40	0.29	0.34	0.95	0.44
11	165.5	$-CH_2-\underline{CH_2}-CH=CH-$	0.53	0.35	0.42	0.97	0.49
12	167.2	$-CH=CH-\underline{CH_2}-CH=CH-$	0.66	0.40	0.48	0.98	0.57
13	167.8[f]	$-\underline{CH_2}-CH_2CO_2-$					
14	170.2	$-\underline{CH_2}CH_3$	1.4	0.88	0.84	0.42	0.48
15	179.0	$-\underline{CH_3}$	2.8	1.3	1.3	0.43	0.44

[a] An estimate of the error is approximately ±10% for T_1 and ±14% for $1/T_{1N}$. The T_1 values represent averages from three separate experiments which were reproducible within approximately 15%. [b] Corresponds to labeling in Figure 2. [c] In parts per million upfield from CS_2. [d] Sears (1972); Birdsall *et al.* (1972); Stoffel *et al.* (1972); Hamilton *et al.* (1973). [e] Methylene resonances of composite envelope. [f] Shoulder.

Spin-label degradation was estimated by measuring the peak-to-peak amplitude of an esr spectrum of an aliquot from a sonicated EYL dispersion. When precautions were taken to prevent degradation of egg phosphatidylcholine, it was found that the stearic acid spin labels C_n were stable in EYL vesicles for at least 24 hr.

The normal Fourier transform spectra of unlabeled and spin-labeled sonicated EYL dispersions are compared in Figure 2. Resonance assignments made with the aid of model compounds (Birdsall *et al.*, 1972; Sears, 1972; Stoffel *et al.*, 1972; Hamilton *et al.*, 1973) are given in Table I. The ^{13}C nmr spectrum of the olefinic carbon region is resolved into two distinct resonances and the fatty acyl methylene region of the spectrum is resolved into several peaks. Qualitatively, the effect on the EYL resonances due to the presence of the nitroxide spin label is considerably less specific for the C_5- than for the C_{16}-labeled vesicles. For the C_5-labeled dispersion, the relative intensity of the carbonyl resonance, indicated by the arrow in Figure 2b, is decreased more than that of any other peak. The intensity of the choline and the fatty acyl methylene resonances are also decreased. For the ^{13}C nmr spectrum of C_{16}-spin-labeled EYL dispersion, the greatest change in peak height occurs in the terminal methylene and methyl resonances whereas the choline resonance is not significantly perturbed by the C_{16} label (*cf.* Figure 2c).

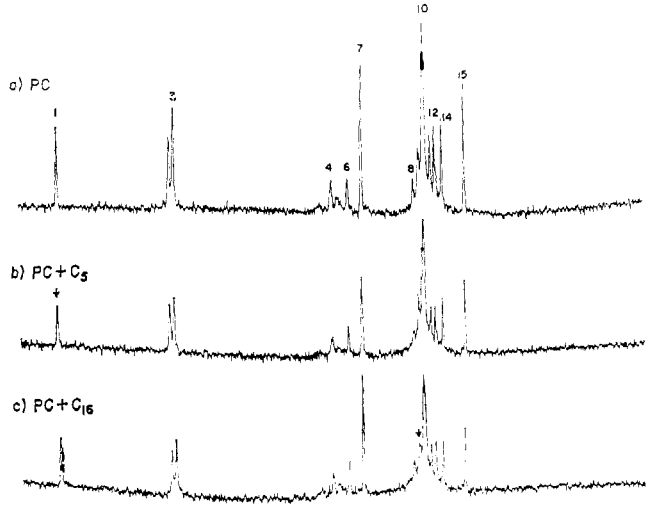


FIGURE 2: Normal Fourier transform spectra of sonicated aqueous phosphatidylcholine (PC) dispersions of egg yolk phosphatidylcholine (PC) (2048 accumulations). (a) Unlabeled vesicles, (b) vesicles labeled with the C_5 stearic acid derivative spin label, and (c) vesicles labeled with the C_{16} spin label. The arrow indicates the resonance whose relative amplitude is most decreased by the particular spin label. The chemical shift range shown is 5–250 ppm upfield from CS_2.

To calculate spin–lattice relaxation times, PRFT sets were obtained. For each partially relaxed resonance, the amplitude A, is given by (Farrar and Becker, 1971)

$$A = A_0[1 - 2\exp(-\tau/T_1)] \quad (1)$$

where A_0 is the equilibrium amplitude, τ is the delay time between the π and $\pi/2$ pulses, and T_1 is the spin–lattice relaxation time. Accurate amplitude measurements in the region of the fatty acyl methylene resonances are difficult due to the overlap of several peaks. To extract accurate amplitudes for this region of the spectrum, the ^{13}C normal Fourier transform nmr spectra were computer simulated, using Lorentzian line shapes. It was found that within the quoted error limits it was sufficient to measure the peak amplitudes directly from the experimental spectra. A least squares analysis of $\ln(A_0 - A)$ *vs.* τ was used to calculate the spin–lattice relaxation time T_1. The results are given in Table I. Within experimental accuracy, no departure from linearity in the $\ln(A_0 - A)$ *vs.* τ plots was found. Lecithin concentration over the range of 0.1–0.2 g/ml, changes of pH from 2.5 to 8.0, and the choice of the buffering system (phosphate-buffered saline or Tris) did not have a significant effect on the ^{13}C nuclear relaxation times.

Levine *et al.* (1972a) have demonstrated that above the gel → liquid crystalline phase transition the ^{13}C T_1 values of sonicated dipalmitoyllecithin dispersions increase with increasing temperature. Therefore, NT_1, where N is the number of protons bonded to a given carbon, is inversely related to the correlation time for rotational reorientation of the carbon nucleus (Abragam, 1961; Allerhand *et al.*, 1971a). In Figure 3, a plot of NT_1 *vs.* approximate fatty acyl hydrocarbon chain position is given. Because of the heterogeneity of the EYL esterified fatty acids, there is some ambiguity in the exact position of the carbon nuclei on the fatty acyl chain. For sonicated egg phosphatidylcholine dispersions, NT_1 increases as the methyl end of the chain is approached, indicating that there is an increase in the motional freedom along the lipid fatty acid chain in going from the carbonyl group to the terminal CH_3. These observations are in agreement with ^{13}C relaxation studies of dipalmitoyllecithin and dioleyllecithin dispersions (Levine *et al.*, 1972a; Metcalfe, 1972). The points corresponding to the olefinic resonances seem to be lower than the general trend, possibly reflecting the lack of free rotation about the double bond. Similarly, there is an increase in NT_1 and hence an increase in the motional freedom in going from the glycerol backbone to the choline group.

It can be assumed that the inverse of the spin–lattice relaxation time of a particular nucleus in a spin-labeled dispersion $(1/T_1)_n$ is the sum of two terms, one due to the relaxation mechanisms occurring in the absence of the spin label, $(1/T_1)_0$, and the other due to the effect of the unpaired electron of the nitroxide, $(1/T_{1N})_n$, such that

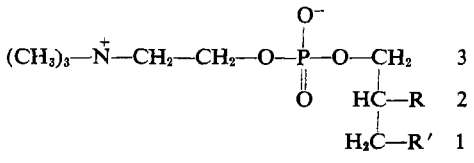
$$(1/T_1)_n = (1/T_1)_0 + (1/T_{1N})_n \qquad (2)$$

where the subscript $n = 5$ or 16 corresponds to the appropriate stearic acid spin label. From eq 2, $(1/T_{1N})_n$ may be calculated and the results are given in Table I.

A plot of the parameter $(1/T_{1N})_n$ *vs.* fatty acyl chain position for $n = 5$, 16 is given in Figure 4. For the C_5-labeled phosphatidylcholine dispersion, the largest effect of the nitroxide on the ^{13}C nuclear spin–lattice relaxation times occurs in the vicinity of the carbonyl group. In addition, the terminal methylene and methyl groups are significantly affected by the C_5 spin label. The largest effect of the C_{16} nitroxide label occurs in the neighborhood of the terminal methylene groups. The middle portion of fatty acyl chain is not affected as much as in the case of the C_5 spin label. There is essentially no effect of the C_{16} nitroxide label on the choline methyl groups. Thus the $(1/T_{1N})_n$ data are in agreement with the qualitative observations made above concerning the effects of the stearic acid derivative spin labels C_n on the relative peak amplitudes.

Discussion

Egg yolk phosphatidylcholine offers several advantages as a model lipid membrane system for nmr studies. The structure of EYL is



where the predominant esterified fatty acids occurring at the 1 position are palmitic (16:0) and stearic (18:0), and at the 2 position are oleic (18:1) and linoleic acid (18:2) (Tattrie

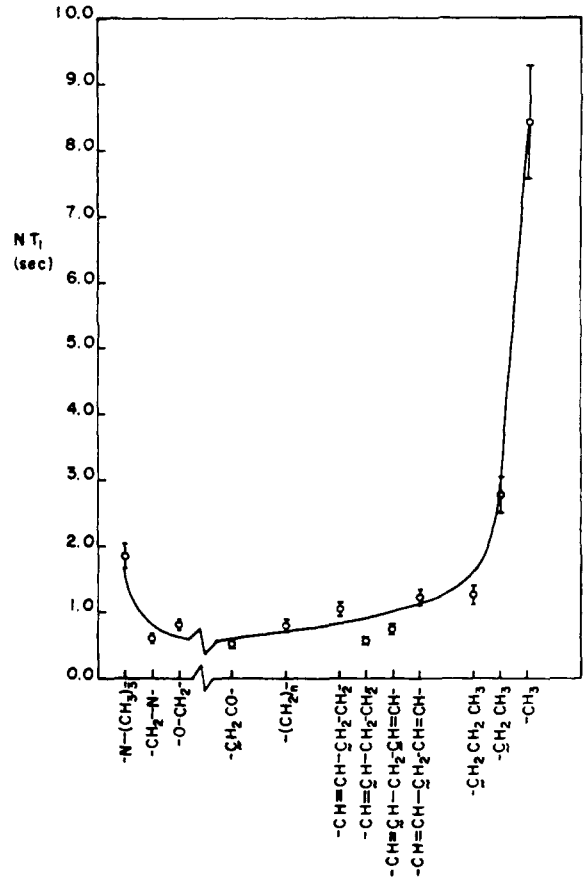


FIGURE 3: Plot of NT_1 *vs.* approximate fatty acyl chain position where N is the number of hydrogens bonded to the carbon nucleus. There is a slight ambiguity associated with the position of a number of carbon nuclei along the fatty acyl chain since egg lecithin contains a heterogeneous distribution of esterified fatty acids.

et al., 1968; Kornberg and McConnell, 1971b). The presence of double bonds in the central region of the hydrocarbon chain gives rise to ^{13}C nmr resonances which are not observed in fully saturated lecithins (Levine *et al.*, 1972a; Stoffel *et al.*, 1972). EYL also contains small amounts (~12%) of long-chain (20 and 22 carbons) esterified fatty acids with varying degrees of unsaturation (Kornberg and McConnell, 1971b). Thus there is some uncertainty in the location of the environment reflected by several resonance peaks including that of the main fatty acyl methylene envelope. The gel → liquid crystalline phase transition temperature for EYL is −5° (Ladbrooke and Chapman, 1969) in contrast with, for example, dipalmitoyllecithin for which the transition occurs at 41° (Ladbrooke and Chapman, 1969). Therefore, the EYL bilayer at room temperature is in the liquid crystalline state corresponding to the state in which a substantial fraction of the lipid in biological membranes is found (Steim *et al.*, 1969; Engelman, 1970, 1971; Overath *et al.*, 1970; Reinert and Steim, 1970; Wilkins *et al.*, 1971; Blazyk and Steim, 1972; McConnell *et al.*, 1972; McFarland, 1972).

The data in Figure 3 demonstrate that the lecithin bilayer is characterized by a marked increase in motional freedom away from the relatively immobilized glycerol backbone toward the choline and the terminal methyl moieties. This trend is in agreement with earlier spin label (Hubbell and McConnell, 1971; Jost *et al.*, 1971) and nmr studies (Birdsall *et al.*, 1971b; Metcalfe *et al.*, 1971; Chan *et al.*, 1972; Horwitz *et al.*, 1972; Lee *et al.*, 1972; Levine *et al.*, 1972a,b; Metcalfe, 1972). Recently, Horwitz *et al.* (1973) have suggested

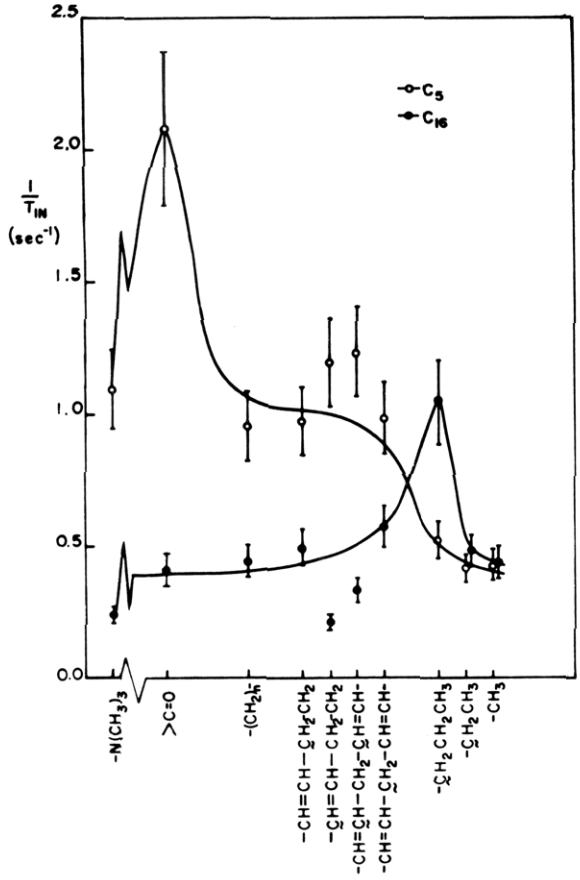


FIGURE 4: Plot of $1/T_{1N}$ *vs.* approximate fatty acyl chain position for egg yolk phosphatidylcholine dispersions labelled with the C_5 and C_{16} spin labels.

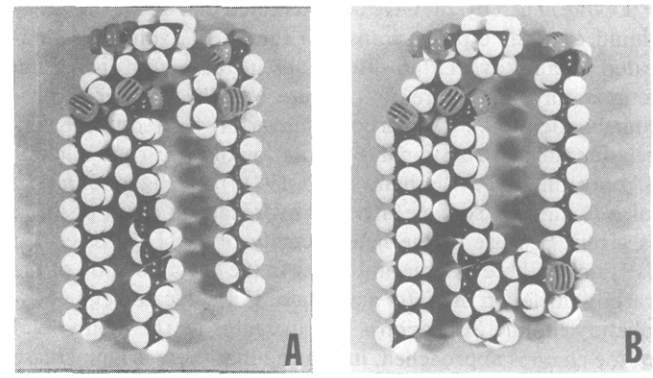


FIGURE 5: CPK models of phosphatidylcholine (with palmitic and linoleic acids as the esterified fatty acids) and the stearic acid derivative spin labels C_5 and C_{16} illustrating (A) the positioning of the nitroxide ring for the C_5 spin-labeled dispersion, and (B) the positioning of the nitroxide ring for the C_{16} spin-labeled dispersion. For simplicity, the fatty acyl chains are shown in the extended rather than the bent configuration suggested by McFarland and McConnell (1971).

that although there is qualitative agreement between esr and nmr measurements of the rigidity gradient, the proton T_1 values reflect positional fluctuations while the esr data reflect a time average ordering at the position of the nitroxide.

From the data given in Figure 4, the position and the orientation of the stearic acid derivative spin labels in the phosphatidylcholine membrane can be deduced. The largest perturbation (*i.e.*, the largest $(1/T_{1N})_5$) of the ^{13}C nmr spectrum of C_5 spin-labeled vesicles occurs at the carbonyl peak. Similarly, the largest effect of the C_{16} label is detected at the $-CH_2CH_2$-CH_3 peak. It is reasonable to assume that the maximum value of $(1/T_{1N})_n$ corresponds to the nitroxide ring of the spin label being on the average in the closest proximity of that given carbon of the phospholipid. Accordingly in Figure 5A, CPK models of EYL and the C_5 stearic acid label are shown with the spin label positioned such that its nitroxide group is located next to the phospholipid carbonyl group. Similarly in Figure 5B, the CPK model of the C_{16} spin label is aligned next to the EYL model with the nitroxide ring placed in the vicinity of the penultimate CH_2 of the phospholipid fatty acyl chain. A comparison of the position of the C_5 and C_{16} spin labels in Figure 5A,B indicates the remarkable consistency in the positioning of the carboxylic acid group of the spin label in the vicinity of the lecithin phosphate group. Since near the lecithin carbonyl group there are no resonance markers for which very accurate $(1/T_{1N})_5$ values can be obtained, it is difficult to be absolutely certain about the precise position of the C_5 spin label. However, because of the extremely large effect of the C_5 label on the lecithin carbonyl group, and because of the appreciable perturbation of the choline methyl groups by the C_5 label the placement of the nitroxide of the C_5 spin label next to the carbonyl group seems appropriate. The agreement in the positioning of both the C_5 and C_{16} labels would indicate that the polar character of the nitroxide ring is not a major factor in determining the position of the stearic acid spin labels. These results suggest, therefore, that the negatively charged carboxyl group of the stearic acid spin label is located in the approximate vicinity of the negatively charged phosphates of the phosphatidylcholines, and that the C_5 label probes the environment just below the glycerol backbones of the phospholipids. However, since the detailed conformation of the polar head groups is not known, the positively charged trimethylammonium groups may also be in close proximity to the carboxylic acid group of the spin label.

If it is assumed that the phospholipid spin labels, containing a nitroxide modified fatty acyl chain, are positioned within the membrane in the same way as other phospholipids, the results of the positioning of the C_5 stearic acid label would imply that the environment probed by the C_n label would be more rigid than that seen by the analogous phospholipid spin label. It has been shown, however, that the environment of the stearic acid derivative label is more fluid than that of the corresponding phospholipid label in EYL–cholesterol vesicles (Hubbell and McConnell, 1971). If the position of the C_n label is independent of cholesterol content, the apparent contradiction can be explained by assuming that the motion of the phospholipid fatty acyl chain is sterically hindered by the proximity of the other chain bonded to the 1 position of the glycerol moiety. It might, therefore, be suggested that in the lipid bilayer region near the glycerol backbone the phospholipid derivative spin label reflects the motional freedom of the individual fatty acyl chains of the phospholipids whereas the stearic acid label reflects the bulk fluidity of the lipid bilayer.

The $(1/T_{1N})_n$ data (*cf.* Figure 4 and Table I) reflect the relative efficiencies of the C_n spin label as a paramagnetic relaxer of the ^{13}C nuclei at given positions on the EYL molecule. The following trends can be discerned. (1) For the C_5 spin-labeled vesicles, $(1/T_{1N})_5$ decreases from the carbonyl group to the terminal methyl group. The C_{16}-labeled vesicle data indicate that the largest effect is at the $-CH_2CH_2CH_3$ position and that the perturbation of the spin label decreases very rapidly away from this group. (2) The C_5 spin label has

an effect on the terminal methylene and methyl groups approximately equivalent to that of the C_{16} label. (3) The ^{13}C nuclei located in the middle of phospholipid fatty acyl chain are perturbed to a greater extent by the C_5 spin label than by the C_{16} label. (4) The maximum in $(1/T_{1N})_5$, which occur at the carbonyl group, is greater than the maximum in $(1/T_{1N})_{16}$, which occurs at $-CH_2CH_2CH_3$. (5) The choline methyl groups are significantly affected by the C_5 spin label but not appreciably by the C_{16} label. (6) The points in Figure 4 corresponding to the olefinic carbons do not appear to follow an obvious trend. This may possibly be due to the inappropriateness of applying eq 2 to these carbons or to the rotational immobilization about the double bond.

Since no adequate theory has been developed for analyzing the ^{13}C nuclear relaxation mechanisms in terms of the anisotropic motion of the fatty acyl chains in lipid bilayers, it is not possible at the present time to quantitatively interpret the $(1/T_{1N})_n$ data. The quantity $(1/T_{1N})_n$ will be a function of the electron–nuclear correlation time. It is also clear that $(1/T_{1N})_n$ will increase as the distance between the nitroxide moiety and a given ^{13}C nucleus decreases. If it is assumed that the major features of the $(1/T_{1N})_n$ plot (*cf.* Figure 4) reflect the proximity of the nitroxide moiety to a given ^{13}C nucleus, then the following conclusions can be drawn. Observations 1, 2, and 3 indicate that the fatty acyl methylene chains of both the spin label and lecithin are quite fluid. The lecithin hydrocarbon chains swing up toward the polar head group region of the membrane (*cf.* points 1, 2, and 3) and consequently the lateral diffusion of the lipids in the plane of the membrane is accompanied by a "whipping" motion of the fatty acyl chains. The hydrocarbon chain of the stearic acid derivative spin label, however, does not apparently swing up as high as the EYL fatty acyl chains (*cf.* points 1, 2, and 3). This is consistent with spin-label esr (Rottem *et al.*, 1970; Tourtellotte *et al.*, 1970) and nmr (Levine *et al.*, 1972) studies which have shown that the fluidity of a bilayer is increased as the degree of fatty acyl chain unsaturation increases. The greater amplitude of the "whipping" motion of the EYL hydrocarbon chains in comparison with that of the stearic acid derivative spin label may reflect the looser packing required by the unsaturated fatty acyl chains of EYL. We cannot, however, exclude the possibility that the difference in the motion of the saturated fatty acyl chain of the spin label and the unsaturated chain of the phospholipid may at least in part be due to the extra mass of the nitroxide ring.

The esr spectra of C_{16}-labeled lipid membranes indicate a high degree of motional averaging of the hyperfine coupling tensor as a result of a very fluid environment. The suggestion that the amplitude of the "whipping" motion of the fatty acyl chain near the nitroxide moiety is limited indicates that the motional averaging is due to relatively rapid but somewhat localized fluctuations in the orientation of the nitroxide ring.

The NT_1 data (*cf* Figure 3) indicate that the trimethylammonium group of the choline moiety is relatively mobile. Furthermore, significant coupling is observed between the C_5 spin label and the choline methyl groups (*cf.* point 5). These results suggest that the choline–phosphate dipole is not rigidly oriented perpendicular to the membrane surface and that the trimethylammonium groups spend some time in the vicinity of the glycerol backbone. (One possible configuration is shown in Figure 5.) This configuration, in addition to internal salt linkages as suggested by Shah and Schulman (1967), would reduce the charge repulsion inherent in the choline–phosphate dipole oriented rigidly perpendicular to the membrane surface. Since the C_4 stearic acid derivative spin label greatly perturbs the proton resonance of the lipid trimethylammonium group of sarcoplasmic reticulum membranes (Eletr and Inesi, 1972), the orientation of the choline moiety in this system may be similar to that in the EYL preparations.

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