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THE MOLECULAR BASIS OF FLUIDITY IN MEMBRANES*

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Fluidity as a prominent feature of the phospholipid portion of biological membranes, as well as of model phospholipid bilayer systems, has been detected by numerous physical techniques[1]). However, correlation of this fluidity with biological functions of membranes is, as yet, documented in only a few cases. For example, fatty acid auxotrophs of *E. coli* grown on different fatty acids exhibit an abruptly increased rate of transport of metabolites across the cell wall at temperatures above the "melting" temperature of the fatty acid supplement[2,3]). The physical properties of lipids extracted from *E. coli* also reflect the temperature at which the bacteria were grown[4]). Fluidity of hydrocarbon chains has been related to the calcium dependent ATPase activity of sarcoplasmic vesicles[5]). A number of other essential functions of biological membranes may very well be associated with fluidity[6,7]), but such considerations are limited by lack of precise knowledge of the molecular basis of fluidity and of the rates of motions involved. The following discussion will review the use of spin labels[8–11]) to determine the rates of several of the motions involved in the fluidity of phospholipid bilayers and, where possible, to provide a structural basis for these motions.

I. Amphiphilic spin labels undergo similar motions in both preparations of biological membranes and in phospholipid bilayers

Among the spin labels used to compare the motions in fluid regions of biological membranes with motions in phospholipid bilayer model systems are a small molecule, TEMPO I, a steroid II and fatty acids III (*m*, *n*).

When I is incorporated into rabbit vagus nerves, unmyelinated walking leg nerve fibers of lobster[12]), and sarcoplasmic vesicles[13]), the resulting paramagnetic resonance spectra indicate that TEMPO (I) has partitioned between the aqueous medium bathing the sample and a low viscosity, hydro-

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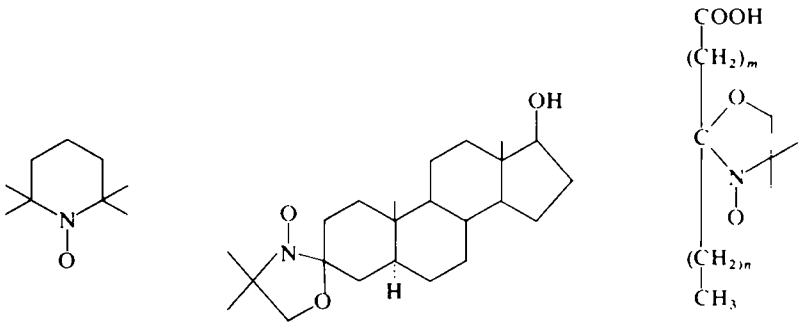


I II III (m, n)

carbon-like region. TEMPO moves rapidly and essentially isotropically in both environments. For this rapid rotation the molecular coordinates of the spin label molecule are averaged, and the upper limit to the correlation time, τ_2, for this rotation may be approximated by

$$\tau_2 \lesssim \frac{1}{2\pi(T_{zz} - T_{\text{isotropic}})},$$

where T_{zz} ($\sim$90 MHz) is the largest component of the hyperfine splitting due to anisotropic electron-nuclear interaction, and $T_{\text{isotropic}}$ ($\sim$42 MHz) is the average of components of the hyperfine interaction. Thus, TEMPO rotates in a hydrocarbon-like fluid in a membrane with a correlation time less than 0.5×10^{-9} sec. The fact that a liquid hydrocarbon-like region exists in membranes strongly implies that some portion of the hydrocarbon chains of lipids is highly flexible.

On the other hand, paramagnetic resonance spectra indicate that the motion of the amphiphilic labels II and III is restricted relative to the motion of TEMPO[14,15]. For molecules as small as II and III, restricted motion cannot reasonably be atrributed to a decreased rate of isotropic motion of the molecule but must arise from restricted movement about one or more molecular coordinates (anisotropic motion)[15]. This can occur if a small molecule is "bound" to a larger structure which has a motional life-time long relative to the correlation time of the small molecule rotating freely in solution. Thus, in addition to the highly fluid regions in membranes, there must be some long range structural features which have a life-time considerably longer than 0.5×10^{-9} sec.

The nature of the binding of steroid and fatty acid spin labels to membranes can be inferred from the effects of structure on the anisotropic motions of the labels. When the polar hydroxyl group of label II is replaced by hydrogen, the spectra of this steroid label in the walking leg nerve fiber

of lobster as well as in sonicated dispersions of phospholipids indicate a slight increase in the overall motion (or a decrease in the anisotropy of motion) of this label relative to the motion of II[14]).

Fatty acid labels, III(m, n) have been synthesized with the spin label at varying positions on the acyl chain[16,17]). The freedom of motion of these spin labels in membranes and phospholipid bilayers increases with increasing the distance from the polar carboxyl group. When lipids are extracted from membranes, labels II and III no longer bind to fluid regions[18,5]). One may conclude then that a polar functional group "anchors" II and III in a region of membranes where an aggregate of phospholipids has a relatively long-lived, long-range structure.

There is now little doubt that phospholipid bilayers are present in biological membranes as well as in pure lipid-dispersions[19]). Since labels I–III exhibit similar motional freedom in both preparations, and this motion is dependent on label structure, it is reasonable to conclude that the motions reflect a bilayer structure. For accurate analysis of the possible types of motions in bilayers, pure phospholipid bilayer preparations have been used to avoid uncertainties due to possible effects of heterogeneity in biological membranes. A recently developed assay for the portion of phospholipids which are in a fluid state in biological membranes indicates that the fluid portion is large[13]), in at least some membranes, and thus pure phospholipid bilayers appear to be a valid model for fluidity in biological membranes.

II. Spin label spectra may be used to measure a wide range of motions in phospholipid bilayers

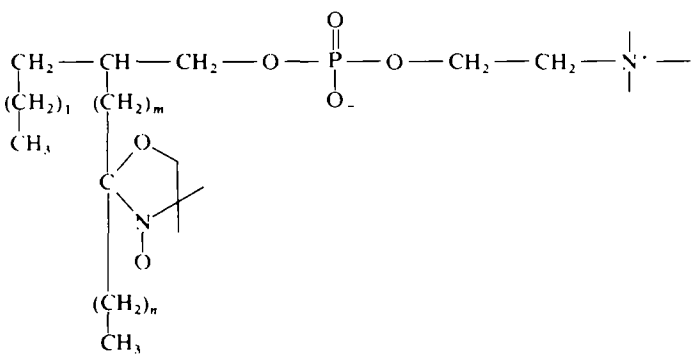
Paramagnetic resonance spectra arising from nitroxide spin labels are motion dependent[8,9,17,20,21]), orientation dependent[8,9,15,22–25]) and concentration dependent (here, concentration dependence refers either to the effect of local concentration of paramagnetism on the spectra[26]) or to the total integrated intensity of spin label signal[27])). By taking advantage of all of these factors controlling spin label spectral features, it is possible to measure a range of motions occuring anywhere from nanoseconds to hours. The following examples were chosen because, viewed as a whole, they offer a detailed description of the "structure of fluidity" in membranes, while, at the same time, they are representative of the numerous possible approaches to measurement of rates by the spin label technique.

A. Motion of phospholipid acyl chains

The spectral line shapes of nitroxides, for which the hyperfine splitting is in the 10–100 MHz range, are sensitive to motions occurring from $\sim 10^7$ to

$> 10^9$ times/sec[8,28]). When the labels undergo isotropic motion, the spectra may be used for accurate measurement of rotational correlation times in this range[20,21]). For anisotropic motion of labels, however, the spectra depend on the amplitude of the anisotropic motion as well as on one or more correlation times. In this case, rapid movement about the molecular coordinate of least motion produces a time-averaged spectrum and the amplitude of this motion is described by the order parameter, S[29,17]). The order parameter of a solid is 1 and of an isotropic liquid is 0.

When spin labeled fatty acids, III (m, n), are incorporated in smectic liquid crystals[29]), the amplitude of motion about the long axis of the chain increases as the distance between the polar head group and the spin label increases. In this case, there is a *linear* decrease in log S with increasing n, the number of methylene groups separating the label and the head group. The linear relationship is consistent with a model[17,29]), which assumes rapid motion about the chain axis as a result of *trans-gauche* isomerizations which occur with equal probability at each methylene position. In such a model, there is, however, a problem of reconciling the increasing motion with an array of parallel packed chains[22]). Furthermore, when these fatty acid labels or lecithins esterified with spin label acyl chains, IV (m, n), are incorporated into bilayers of egg lecithin, the characteristic decrease in order parameter is *greater than logarithmic*[17,22]).



IV (m, n)

For these cases, the *trans-gauche* isomerization model must be modified to include other effects such as increased probability of occurrence of *gauche* conformers in the terminal methyl region of the chain due to a change in the time-average direction of chain orientation. For instance, the average axis direction might be perpendicular to the bilayer plane throughout part of the chain length, but bent from this direction in another region. The near absence of low angle diffraction of X-rays from chains in bilayers, indicates that any

change of chain orientation certainly must be gradual throughout the bilayer, not abrupt[30]). If a bending of the chains were present, one would expect the orientation-dependent spin label spectra to reflect it.

The orientation dependence[15,22-25]) of paramagnetic resonance spectra of III(*m*,*n*) and IV(*m*,*n*) arises from the anisotropic electron-nuclear interaction of nitroxides, the fixed geometry of the nitroxide coordinates relative to the long axis of the extended polymethylene chain, and the orientation of the sample in the magnetic field. The spectra may be analyzed in oriented bilayers because *trans-gauche* isomerizations are rapid enough that the spectra reflect the average orientation of the long axis of the chain.

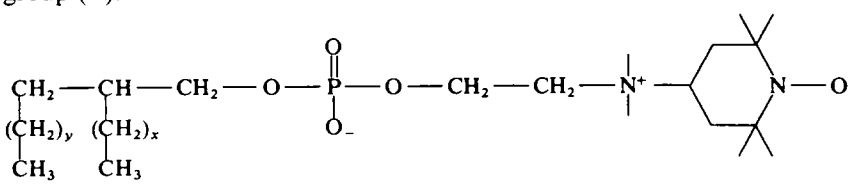
In fact, when phospholipid labels IV(*m*,*n*) are incorporated into oriented multilayers of hydrated egg lecithin, the time-average orientation of the chains in the terminal methyl region is found to be perpendicular to the bilayer surface, but the most probable orientation is bent from this direction by an angle of about 30° near the group[22]). This net tilt of the lipid chains near the head group is observed in both egg-lecithin[22]) and egg lecithin-cholesterol (2:1)[31,32]) multilayers with spin labeled acyl chains on either the β-[22,31]) or γ-[32]) positions of lecithin. Spectral calculations indicate that the tilted region must have a life-time long compared to $\sim 10^{-8}$ sec.

If chain orientation is to serve as a structural basis for observed motions of acyl chains, then it must provide an explanation of the greater than exponential differential drop in order parameter. An idealized model[22]) of chain packing depicts a cluster of neighboring chains perpendicular to the bilayer plane in the methyl-terminal half of the chain and bent in the region of the head group. Although the time-average area occupied by the chain segments in upper and lower portions of the chains is the same when projected onto the bilayer plane, the model implies a difference in carbon atom density between the two regions. Such a difference in density of chain packing would give greatest freedom of motion to the terminal methyl portion of the chain. The apparent density difference is somewhat diminished, however, by a "back-flow" of carbon atoms resulting from the finite probability that segments of the chain have an instantaneous orientation different from their time average orientation[31]). The probability distribution of the instantaneous orientation of chains is of interest for comparison with X-ray scattering evidence for chain orientation[30]). Calculations of this probability distribution are currently in progress. The calculations consider both the long-time average orientation of a segment of acyl chain as well as the amplitudes of rapid motions about the axis of orientation.

B. Phospholipid exchange across the bilayer

As indicated earlier, the observed anisotropic motion of the amphiphilic

spin labels in bilayers implies that some features of the bilayer structure are long-lived relative to the rate of acyl chain isomerization. The slow exchange of phospholipids between two sides of the bilayer is a minimum requirement for such a long range structure. In fact, the rate of exchange of phospholipids across the bilayer has been measured and is exceedingly slow, that is, slower than 2×10^{-5} times/sec[27]). The measurement of such a slow rate does not depend on spectral line shape parameters, but on the change in total concentration of spin label in the sample. The change in concentration is brought about by selective chemical reaction of spin labels on the outside of the vesicles. Rapid reduction of the paramagnetic nitroxide group, and concomitant loss of signal, is accomplished by adding ascorbic acid to chilled aliquots of vesicles containing low concentrations of lecithin spin labeled in the head group (V).



V

At 0° ascorbic acid does not penetrate the vesicles and reduces only those spin labels on the outside, and no equilibration of labels occurs. In this way, vesicles with label only on the inside are prepared. Aliquots, taken at different times from the vesicle preparation equilibrating at 30°, are cooled to 0° and treated with ascorbic acid to reduce any label which has exchanged from inside to outside. Thus, the rate of exchange of lecithin between the inside and outside of the vesicle was measured.

The slow rate of inside-outside exchange has a number of implications for the structure and function of biological membranes. For instance, an assymetric membrane may be maintained with essentially no expenditure of metabolic energy. Furthermore, it will be of interest to see if the exchange rate is accelerated by transport of specific molecules across membranes.

III. Lateral diffusion of phospholipids in the bilayer plane

The extremely slow rate of exchange across the bilayer implies an almost static bilayer with respect to this process. However, if one considers the rapid chain isomerizations in the hydrophobic interior of the bilayer, occurring more than $\sim 10^8$ times/sec, it is clear that a bilayer is far from static from this point of view. Therefore, there is little basis for predicting the rate of lateral diffusion of molecules within one bilayer plane[26]).

It was possible to set a limit on the rate of lateral diffusion by investigating the interaction of the paramagnetic spin label (V) with proton resonance signals of unlabeled lecithin. All of the signals of the N-methyl protons of choline headgroups are broadened equally by their interaction with paramagnetic head groups. Measurement of the dependence of broadening on spin label concentration and temperature indicated that the frequency of molecular jumps leading to lateral diffusion was considerably greater than 3×10^3 jumps per second. That is, the lifetime of a molecule between its neighbors is less than 10^{-4} sec[33]).

Another method of getting at the rate of lateral diffusion is to investigate the diffusion of a small, highly concentrated patch of spin-labeled lecithin into a surrounding unlabeled bilayer. The measurement in this case depends on the dramatic spectral differences arising from a local high concentration of spin label as compared with a low concentration of label. Analysis of the time dependence of spin label concentration as label diffuses from a concentrated patch in oriented multilayers of hydrated lecithin demonstrated that the rate of lateral diffusion is considerably more rapid than the limit set by the nuclear magnetic resonance measurements. The measured diffusion constant was 1.8×10^{-8} cm^2/sec, or a lifetime of a molecule between neighbors of $\sim 10^{-7}$ sec[26]). The rate of lateral diffusion is, then, at least two orders of magnitude less than that of chain isomerizations near the center of the bilayer. However, there is some indication that the diffusion rate is related to the amplitude of these chain motions because, as mentioned earlier, the fluidity of the hydrocarbon chains is considerably restricted, especially near the head groups, by the introduction of cholesterol. Likewise, the rate of diffusion in equimolar mixtures of lecithin-cholesterol is decreased by a factor of 1.5–2.0[26])

The concentration broadening of a spin label signal, in this case of spin labeled cholesterol, has been used in quite a different approach to measure the rate of lateral diffusion[34]). In this case the measured diffusion constant was $\sim 10^{-8}$ cm^2/sec.

A. An assay for the extent of fluid phospholipid regions in biological membranes

The measured motions in phospholipid bilayers have numerous implications for the mechanism of biological membrane function. However, one of the first questions to arise in such considerations is that of what fraction of the total membrane phospholipid has the fluidity properties which have been measured in pure phospholipid bilayers. Preliminary experiments constituting an assay for the extent of fluid regions indicate that in the sarcoplasmic reticulum membranes, more than half of the lipid is in the fluid state detected by spin labels[13]).

This assay depends on the partitioning of TEMPO I between aqueous medium and fluid hydrophobic regions of a membrane. The partition coefficient of TEMPO depends on the average fluidity of the hydrophobic region and the fatty acid labels III (m, n) may be used to measure this average fluidity. The partition coefficient of TEMPO at a particular fluidity is determined for pure lipid vesicles in which the fluidity has been adjusted, by proper mixture of lipids, to that of the biological membranes. This partition coefficient is then used to analyze the TEMPO spectrum of a sample of biological membrane having a known weight of total lipid.

The order parameter plot of spin label fatty acid data for sarcoplasmic vesicles is typical of these labels in fluid bilayers[5]). Applying the above assay to these vesicles, we find that $\sim 84\%$ of the membrane phospholipids are in fluid regions.

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Discussion

SEEMAN: Dr. C. Hsia and Dr. L. Spero at the Department of Pharmacology, University of Toronto, are currently measuring the intramembrane translocation of a spin-labelled molecule, myristicamide [N-(1-oxy-2, 2, 6, 6-tetramethyl-4-piperidinyl) myristate]. The spin-labelled myristicamide is added to intact erythrocytes or erythrocyte ghosts and essentially all the compound adsorbs onto the membranes. The ESR spectrum shows that myristicamide is not in free solution but highly restricted in its freedom. The field setting is adjusted to the value of the M_0 peak of the nitroxide ESR spectrum (32190 Hz) and the intensity of the peak signal measured as a function of time. As the myristicamide "flips" over within the bilayer regions of the membrane, the nitroxide is immediately reduced by the endogenous glutathione (3 mM) in the cytoplasm, or by the ascorbic acid inserted into the erythrocyte ghosts by standard techniques.

At 37 °C the inward rate of myristicamide flipping is about 34 min (half-time) for intact cells and about 26 min for ghosts. The outward flipping rate, however, is much faster, about 3.5 min in ghosts. The flipping rates, therefore, are asymmetrical. This asymmetry is maintained if the ghosts are turned inside-out by the procedure of Steck, Weinstein and Wallach. The inward flipping rate of everted ghosts is 3.8 min, while the outward rate is 11 min. Control experiments, where the ghosts are merely made into smaller ghosts have the same rates as normal ghosts, so that it is not a matter of the size of the ghost. Hsia and Spero have also found that after neuraminidase treatment the inward and outward flipping rates are about equal, about 3–4 min.

BANGHAM asked how labelled phospholipid was added to multimembrane myelin system.

MCFARLAND said that in those experiments, fatty acid label, not phospholipid, was added using Bovine serum albumin.

LUZZATI mentioned fluorescent label experiment using a long stiff molecule with transition moment parallel to the length of the molecule added to phospholipid bilayers showing a random arrangement, orientation of lipid chains in the Lα and liquid crystalline phase are not defined.

CHAPMAN mentioned that a series of homologous phospholipids gave X-ray long spacings from which an *apparent* angle of tilt of chains of some 30 or 40 could be deduced.

MEHLHORN asked about the order parameter in phospholipid and egg lecithin systems and also wondered how the presence of double bonds effected the models put foreward.

MCFARLAND said although there may be a small dependence of order parameter on position of unsaturation, the general features of order parameter plots are the same for egg lecithin, dioleyl lecithin and dipalmitoyl lecithin above the transition temperature.

WALLACH: In your esr experiments on vagus nerves, where is the label situated? The vagus nerve membrane is mainly myelin. Even in the so-called unmyelinated nerves such as the squid axon there is a pig sattelite sheet which Schmitt estimates to be 95% of membrane mass of membrane.

MCFARLAND replied that the label is in a site where it experiences considerable motion. If the label were in a position where there was little motion then it should be evident in the spectra, but it is not. We are anxious to apply the assay for % of fluidity to these membranes as we have done with the sarcoplasmic reticulum membrane.

CHAPMAN said it could be that the label goes into a very small but fluid region of the membrane, so that present statements of membrane fluidity from spin label experiments could really refer to the fluidity of only small parts of the membrane structure.

FINER asked how much water is associated with lipids in McFarland's experiments where bent chains are found.

MCFARLAND said that the lipids were fully hydrated and that they had not carried out experiments on dried systems. Their experiments were carried out in systems where the lipids are kept in the presence of water vapour or salt solutions with a relative humidity of 85% or higher.

SEEMAN showed two slides referring to work carried out in the Pharmacology Department, Toronto, describing some flip-flop type experiments, Myristic amide spin label added to outside of ghosts, inside out ghosts and whole red cells. The endogenous glutathione reduces the myristic amide as it

flips over from one side to the other. One can also use ascorbic acid. Dr. Seeman showed results of these experiments (*Write for details), pointing out that the flip in and flip out values differed from normal ghosts, i.e., the membrane was asymmetric. This is confirmed with inside out vesicles.

MCFARLAND asked about leak rate of ascorbic acid in experiments which Dr. Seeman had described. In their own experiments phospholipid vesicles were impermeable to ascorbic acid at 0 °C.

SEEMAN said that Shaw and Spero carried out experiments to check whether glutathione or ascorbate was not leaking out. They used peroide to reduce endogenous glutathione by two or three orders of magnitude and found that the flip-flop rates were the same.

VAN DEENEN noted that the flip-flop rates were much more pronounced in red blood cells than in the lipid vesicle studies. He also pointed out that myristic acid itself was lytic and that the structure of the label used in these experiments was a wedge shaped molecule like lysolecithin. He asked if it was possible to extrapolate results with this spin label to natural phospholipid molecules.

KEITH pointed out that the label used had only the NO group as a polar site and so its anchoring to the membrane will only be small, hence flip-flop across the membrane would be expected to be much greater than with a phospholipid molecule containing a charged group by several orders of magnitude.

CHAPMAN noted that with some spin labels the observation of the marked transition from gel to liquid crystal with dipalmitoyl lecithin depended upon the concentration of spin label used, and asked how this affected conclusions with spin labels in membranes for detecting fluidity or phase transitions.

MCFARLAND said that these effects were observed with fatty acid spin labels and not with phospholipid spin labels.

BLAUROCK compared X-ray diffraction pattern results on oriented lipid systems with the present spin label results. He pointed out that packing as deduced from former is uniform whereas in a bent chain system one would expect chains to be packed more tightly where the chains are tilted to conserve area per molecule.

MCFARLAND said that the real system was more dynamic than indicated by the simple model.