J. Membrane Biol. 31, 131 – 169 (1977)
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Spin-Label Studies on Rat Liver and Heart Plasma Membranes: Do Probe-Probe Interactions Interfere with the Measurement of Membrane Properties?

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Received 18 March 1976; revised 6 July 1976

Summary. The structures of purified rat liver and heart plasma membranes were studied with the 5-nitroxide stearic acid spin probe, *I*(12, 3). ESR spectra were recorded with a 50 gauss field sweep, and also with a new technique which "expands" the spectrum by (1) recording pairs of adjoining peaks with a smaller field sweep and (2) superposing the common peaks. The hyperfine splittings measured from the "expanded" spectra were significantly more precise than those obtained from the "unexpanded" spectra. Both procedures were used to study the effects of various *I*(12,3) probe concentrations on the spectra of liver and heart membranes, as well as the effects of temperature and $CaCl_2$ additions on the spectra of liver membranes, and revealed the following:

The polarity-corrected order parameters of liver (31°) and heart (22°) membranes were found to be independent of the probe concentration, if experimentally-determined low *I*(12,3)/lipid ratios were employed. The absence of obvious radical-interaction broadening in the unexpanded spectra indicated that "intrinsic" membrane properties may be measured at these low probe/lipid ratios. Here, "intrinsic" properties are defined as those which are measured when probe-probe interactions are negligible, and do not refer to membrane behavior in the absence of a perturbing spin label.

At higher *I*(12,3)/lipid ratios, the order parameters of liver and heart membranes were found to substantially decrease with increasing probe concentration. The increase in the "apparent" fluidity of both membrane systems is attributed to enhanced radical interactions; however, an examination of these spectra (without reference to "low" probe concentration spectra) might incorrectly suggest that radical interactions were absent. For the membrane concentrations employed in these studies, the presence of "liquid-lines" (or "fluid components") in the unexpanded ESR spectra was a convenient marker of high probe concentrations.

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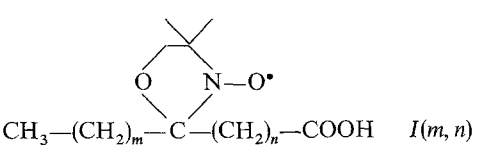
A thermotropic phase separation was observed in liver membranes between 19° and 28°.

Addition of $CaCl_2$ to liver plasma membrane [labelled with "low" $I(12,3)$ concentrations] increased the rigidity of the membrane at 31° and 37°, without inducing a segregation of the probe in the bilayer.

Previously reported data are discussed in relation to these results, and suggested minimal criteria for performing membrane spin label studies are included.

Amphiphilic spin labels have been extensively used recently in the study of the structure and function of biological membranes [10, 16, 23, 35, 38, 49, 71]. Membrane-incorporated spin probes are sensitive to the polarity and fluidity of their local environment. Temperature alterations [9, 10, 38, 49, 51, 63, 66], divalent cation binding [17, 63], and the action of many drugs [27, 30, 69] and hormones [31, 42, 65] perturb labelled biological membranes to yield characteristic changes in their respective ESR spectra. Moreover, comparative studies of different membrane systems with ESR techniques offer the potential for determining (1) pathological membrane structural alterations, and (2) structural properties of functionally different membranes.

An adequate interpretation of ESR spectral changes in terms of the structure of the host matrix requires that alterations in fluidity and/or polarity be distinguished from changes in probe-probe interaction (e.g., dipole-dipole and electron-electron exchange broadening). Probe-probe interactions produce large spectral alterations at "high" probe/lipid molar ratios (i.e., exceeding 1:100), and this effect has been used to estimate lateral lipid diffusion constants in both model and biological membrane systems [13, 14, 60–62, 64]. However, the presence of high local probe concentrations could also interfere with the measurement of membrane fluidity. Hubbell and McConnell demonstrated that the order parameter of $I(10, 3)$-labelled dipalmitoyllecithin decreased markedly as the $I(m, n)$ probe/lipid ratio was increased from 1:600 to 1:100 at 37° [29].



Although it is possible to gain considerable information from membranes using high concentrations of probe, membrane fluidity and polarity are best examined using "magnetically dilute" concentrations.

No detailed studies on the effects of probe concentration on the calculated order parameter of $I(m, n)$-labelled biological membranes have been

previously reported to our knowledge. The tacit assumption is usually made that probe-probe interactions will be minimal if the probe/lipid ratio is kept "low". However, what constitutes a "low" probe/lipid ratio for a given membrane system is usually ill-defined and arbitrary. We report that at probe concentrations which might be considered to be "low", the ESR spectra of *I*(12, 3)-labelled rat liver plasma membrane and rat cardiac sarcolemma reflect probe-probe interactions as well as the fluidity and polarity of the environment of the probe.

The purpose of this study was to examine the effect of wide ranges of probe concentration on the order parameters of *I*(12, 3)-labelled biological membranes, to determine the extent that probe-probe interactions interfere with the accurate measurement of the membrane fluidity, and to establish criteria for assessing if the probe concentration used in a given study permits the measurement of an intrinsic membrane fluidity.

Theory

The paramagnetic resonance spectra of *I*(*m*, *n*)-labelled membranes may be explained quantitatively in terms of an effective Hamiltonian $\mathscr{H}'$ that has axial symmetry, such that

$$\mathscr{H}' = |\beta|\,\mathbf{S}\cdot\mathbf{g}'\cdot\mathbf{H} + h\mathbf{S}\cdot\mathbf{T}'\cdot\mathbf{I} - \mathbf{g}_N\,\beta_N\,\mathbf{I}\cdot\mathbf{H}. \quad (1)$$

Here $\mathbf{g}_N$ is the *g*-factor tensor for the ^{14}N nucleus, β and β_N are the electron and nuclear Bohr magnetons, h is Planck's constant, and $\mathbf{H}$ is the applied magnetic field vector. $\mathbf{S}$ and $\mathbf{I}$ are the electron and nuclear spin angular momentum operators in units of h [29]. The spectrum of *I*(12, 3)-labelled rat liver plasma membrane (Fig. 1*A*) indicates the hyperfine splittings parallel ($T_{\|}$) and perpendicular ($T_{\perp}$) to the unique symmetry axis, z'. Here, z' is one of the principal axes of $\mathscr{H}'$; the remaining principal axes are x' and y'.

The elements of $\mathbf{g}'$ and $\mathbf{T}'$ are the appropriate time averages of the intrinsic (rigid lattice) elements of the *g*-factor tensor and the nuclear hyperfine tensor $\mathbf{T}$. The elements of the static Hamiltonian have been previously obtained by incorporating nitroxide derivatives into host crystals as substitutional impurities. The static values used here to describe the rapid anisotropic motion of membrane-incorporated *I*(*m*, *n*) probes are:

$$(T_{xx}, T_{yy}, T_{zz}) = (6.1, 6.1, 32.4)\,G \quad [67]$$

where x, y and z are the principal axes of the static Hamiltonian; the x axis is parallel to the N–O bond direction, and the z axis is parallel to the nitrogen $2p\pi$ orbital.

If the label undergoes rapid rotational and segmented motion in the membrane, $T_{||}$ may be expressed in terms of the elements of **T** as follows:

$$T_{||} = \overline{\alpha^2}\, T_{xx} + \overline{\beta^2}\, T_{yy} + \overline{\gamma^2}\, T_{zz} \tag{2}$$

$\overline{\alpha^2}$, $\overline{\beta^2}$ and $\overline{\gamma^2}$ are the time averages of the squares of the direction cosines of z' in the x, y, z axis system. This expression is valid if the following conditions are satisfied: (1) the polarity of the environment of the probe in the membrane is identical to that of the probe in the host crystal [i.e., Trace (**T**) = Trace (**T**′)]; and (2) "magnetically dilute" label concentrations are employed, since higher order terms representing probe-probe interactions are not included in $\mathscr{H}'$.

The right side of Eq. (2) may be decomposed into an isotropic and an anisotropic term by noting that $\alpha^2 + \beta^2 + \gamma^2 = 1$, $T_{xx} = T_{yy}$, and by defining an order parameter (S), such that $S = (1/2)(\overline{3\gamma^2} - 1)$:

$$T_{||} = a_N + (2/3)(T_{zz} - T_{xx})\, S. \tag{3}$$

a_N is defined as the isotropic hyperfine coupling constant (in gauss) for the nitroxide molecule in the crystal state, where:

$$a_N = (1/3)(T_{zz} + 2\, T_{xx}). \tag{4}$$

The isotropic hyperfine coupling constant for the probe in the membrane (a'_N) may similarly be evaluated from the elements of **T**′:

$$a'_N = (1/3)(T_{||} + 2\, T_{\perp}). \tag{5}$$

Since Eq. (3) was derived based on the assumption that Trace (**T**) = Trace (**T**′), $a_N = a'_N$. Therefore, the following expression for the order parameter (S') may be obtained, if a'_N is substituted for a_N in Eq. (3):

$$S' = \frac{(T_{||} - T_{\perp})}{(T_{zz} - T_{xx})}. \tag{6}$$

S' was originally derived by Seelig to quantitatively assess the flexibility of the lipid chain. [67]. However, an alternative order parameter (S'') may be derived from Eq. (3) which is a function of $T_{||}$ (independent of $T_{\perp}$) by choosing the isotropic hyperfine coupling constant (a_N) equal to (1/3) $(T_{zz} + 2T_{xx})$. Therefore,

$$T_{||} = (1/3)\,(T_{zz} + 2T_{xx}) + (2/3)\,(T_{zz} - T_{xx})\, S''$$

and solving for S'',

$$S''=(1/2)\left[\frac{3(T_{\parallel}-T_{xx})}{(T_{zz}-T_{xx})}-1\right]. \tag{7}$$

It should be noted that this expression for the order parameter (S'') is identical to that obtained by Ehrenberg and co-workers who assumed a model in which the nitroxide labels were rotating rapidly within the confines of a cone of variable semi-cone angles [33].

The relationship of S' to S'' is of particular interest. S' should be equal to S'' (within experimental error) if the polarity of the membrane is the same as that of the host crystal and probe-probe interactions are negligible. If either of these conditions is not satisfied, S' will not, in general, equal S''. However, appropriate polarity corrections to S' (and S'') may be applied by noting that the isotropic hyperfine coupling constant (a_N) is sensitive to the polarity of the environment of the nitroxide radical. Let us assume that changes in the polarity of the environment of the radical affect T_{xx} and T_{zz} in the same way [67]. Eqs. (6) and (7) may then be corrected by dividing the elements of **T** (and **T'**) by their respective isotropic coupling constants, a_N (and a'_N). Performing this operation on either S' or S'' yields the polarity-corrected order parameter (S) initially derived by Hubbell and McConnell [29]:

$$S=\frac{(T_{\parallel}-T_{\perp})(a_N)}{(T_{zz}-T_{xx})(a'_N)}. \tag{8}$$

The above treatment is valid only when higher order terms representing electron-electron exchange and dipole-dipole interactions make a negligible contribution to $\mathscr{H}'$. We report here that $T_{\perp}$ (but not ($T_{\parallel}$) broadens with increasing $I(12,3)$ concentration in liver and heart plasma membranes at what might be considered "low" probe/lipid ratios; these effects are believed to be due to probe-probe interactions rather than induced membrane structural alterations. Because of the nature of these spectral perturbations, S' and S were observed to decrease with increasing probe concentration, while S'' remained constant. The ratio of S' to S'' (m) is therefore suggested as an intuitively attractive measure of such probe-probe interactions:

$$m=\frac{S'}{S''}=(2/3)\frac{(T_{\parallel}-T_{\perp})}{(T_{\parallel}-a_N)}$$

and also

$$m=\frac{(T_{\parallel}-a'_N)}{(T_{\parallel}-a_N)}. \tag{9}$$

Thus, in the limiting case where the polarity of the membrane is identical to that of the host crystal and radical interactions are absent, $m=1$; if probe-probe interaction effects increase either the $T_{\|}$ or $T_{\perp}$ splitting, m will be less than 1.

If the membrane polarity is not the same as the reference crystal, m may be expanded into an "intrinsic" term (m°) and a term which depends on probe concentration:

$$m = m^{\circ} - \frac{2(\Delta T_{\perp})}{3(T_{\|} - a_N)}.$$

Here, m° is the ratio of S' to S'' measured at "infinitely dilute" probe concentrations, $T_{\|}$ is assumed not to depend on probe concentration, and $\Delta T_{\perp}$ is the broadening of the inner hyperfine splitting due to probe-probe interactions. It is readily seen that positive increases in $\Delta T_{\perp}$ will decrease m; similar arguments may be employed to show that probe concentration-dependent increases in $T_{\|}$ would also decrease the m ratio.

The study of the m ratio as a function of probe concentration for a given membrane system might therefore suggest regions where "intrinsic" properties may be measured. Moreover, the m ratios calculated from earlier $I(m, n)$ studies might indicate whether "high" probe concentrations (i.e., probe concentrations which perturb $T_{\|}$ and/or $T_{\perp}$) were used.

Materials and Methods

Materials

We obtained the N-oxyl-4',4'-dimethyloxazolidine derivative of 5-ketostearic acid, $I(12,3)$, from Syva Co., Palo Alto, California. L-α-(distearoyl) lecithin and sucrose were purchased from Calbiochem, La Jolla, California. Liquid nitrogen storage tubes were obtained from Microbiological Associates, Los Angeles, and stainless steel wire cloth (mesh 40) was from Small Parts, Miami, Florida. Male albino rats were obtained from Holtzman Co., Madison, Wisconsin. All other chemicals were from Sigma Chemical Co., St. Louis, Missouri.

Density Gradients for the Cardiac Membrane Preparation

Sucrose density gradients were made with a Buchler density gradient mixer (5 ml capacity). A Buchler Instruments Auto Densi-Flow II was used to layer gradients of sucrose (30–68.4 % w/v) into nonwettable polyallomer centrifuge tubes. Ten 0.5 ml fractions were collected with the Auto Densi-Flow II, and the sucrose content was estimated with a Zeiss Abbe refractometer at 21.5°.

Analytical Procedures

5'-Nucleotidase, inorganic phosphate and protein were measured as described previously [63]. Phospholipid was determined according to Bartlett [3], employing L-α-(distearoyl) lecithin as a standard.

Filtration Device

Much of the contractile protein of the cardiac preparation was removed with a filtration device similar in principle to one described earlier [39]. A circular stainless steel cloth (40 mesh) was layered into a Buchner funnel (5 cm diameter) and sealed to the funnel with epoxy glue.

Preparation of Plasma Membranes

Rat liver plasma membranes were prepared as reported earlier [63] and suspended in 8% sucrose, 5 mM Tris-HCl (pH 7.6). Liver membrane phospholipid was determined after extracting samples with chloroform/methanol (2:1) for 24 hr. The protein/phospholipid ratio was 1.4 mg/mg, assuming all membrane phospholipid to have a mol.wt. of 780 g/mole.

Cardiac sarcolemma was prepared according to the procedure of Kidwai *et al.* [39, 40] with several modifications. Red blood cells were removed from 5–7 g (wet weight) of rat hearts by thorough washing with 0.25 M sucrose. The tissue was minced and then dispersed in a Potter Elvejhem homogenizer for about 15 sec in 0.25 M sucrose. The homogenate (20% w/v) was filtered under mild suction using the filtration device described above. The filtrate was centrifuged in a Beckman Spinco Ultracentrifuge in a 50 k head at 43,000 rpm for 30 min. The pellets were resuspended in minimal volumes of 0.25 M sucrose. Aliquots (0.6 ml) of this material were loaded onto the sucrose gradient (described above) and centrifuged for 90 min at 40,000 rpm in a Beckman SW 50.1 rotor. Five 1 ml fractions were collected from the tubes with the Auto Densi-Flow II, diluted to 0.25 M sucrose, and centrifuged at $100{,}000 \times g$ for 30 min. The pellets were resuspended in 8% sucrose, 5 mM Tris-HCl (pH 7.6), and the various fractions were assayed for 5′-nucleotidase and protein. Separate aliquots were examined with a Zeiss phase contrast microscope.

Spin Labelling

The *I*(12,3) probe was dissolved in ethanol (10^{-3} M) and various aliquots were dried with a stream of dry N_2 gas in liquid nitrogen storage tubes. 120 µl samples of plasma membrane (3.3 mg liver or 2.3 mg cardiac membrane protein), stored until use at −70°, were then added to the probe and gently vortexed for several min at room temperature. The following spin label/membrane ratios were employed: (1) 0.15–14.3 µg *I*(12,3) per mg of cardiac sarcolemma protein, and (2) 0.44–5.7 µg *I*(12,3) per mg of liver plasma membrane protein (or 1 molecule of probe per 65–775 lipid molecules, assuming all liver membrane lipid to be phospholipid with a ratio of 1.4 mg protein/mg phospholipid). Spectra were recorded in a 0.15 ml capacity aqueous cell with a Varian E-3 ESR spectrometer equipped with a variable temperature accessory. Samples were equilibrated for five min prior to taking spectra. The temperature was calibrated from 15–40° using a thermocouple placed in the resonant cavity. The microwave power was kept at 40 mW unless otherwise indicated.

Spectral Measurements

An ESR spectrum of *I*(12,3)-labelled rat liver plasma membrane, recorded with a 50 gauss field sweep and four min scan time, is shown in Fig. 1*A*. Only the relative positions of the maxima (minima) were used to determine $T_{\parallel}$ and $T_{\perp}$. Errors associated with positioning the peaks in the unexpanded spectrum usually preclude an order parameter (*S*) determination more precise than ±2–3%.

The precision of the hyperfine splitting measurements may be improved if the spectrum is "expanded" (Fig. 1*B*) by recording each pair of adjoining numbered peaks in Fig. 1*A* with a field sweep smaller than 50 gauss. The pairs of peaks 1 and 2, 2 and 3, and 3 and 4 were recorded separately with a field sweep of 10 gauss; the sweep used to record peaks 4 and 5 was

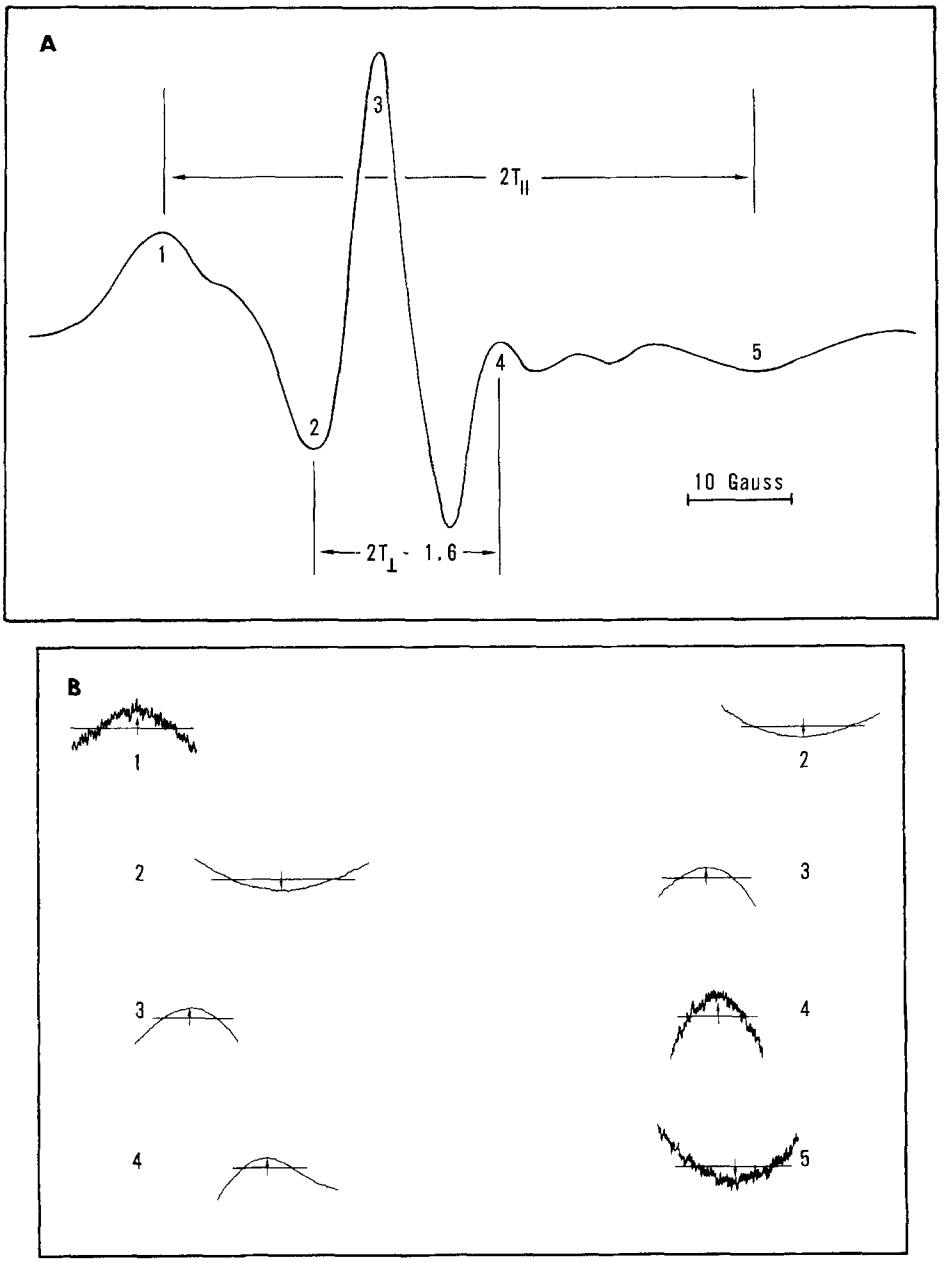


Fig. 1. Spectra of rat liver plasma membrane (31°) labelled with 2.03 μg *I*(12,3)/mg protein. *(A)* Unexpanded spectrum recorded with a 50 G field sweep, 4 min scan time. $T_{\parallel}$ and $T_{\perp}$ are measured as shown; $2T_{\perp}$ is corrected by the addition of 1.6 G [29]. Peak 4 is referred to in the text as the high field peak of the inner hyperfine doublet. *(B)* Expanded spectrum, in which each pair of adjoining numbered peaks in *A* is recorded with a field sweep smaller than 50 G. Peaks 1, 5 and 4 (of the pair 3 and 4) were scanned with a 5×10^5 receiver gain, 16 min scan time; all others were recorded at 5×10^4 receiver gain, 4 min scan time. *See* Material and Methods for the measurement of $T_{\parallel}$ and $T_{\perp}$ from the expanded spectrum

25 gauss. Magnetic field strength settings were selected to center each pair of peaks on the chart paper (Fig. 1*B*). Broad maxima (minima) were scanned with a 5×10^5 receiver gain, 16 min scan time; all others were recorded at 5×10^4 receiver gain, 4 min scan time. The position of each peak was determined from the intersection of a horizontal line with the curved portion of the trace; the mid-distance between the two intersection points was defined as the peak position. If the peak lineshape was symmetrical, the positioning of the peak was insensitive to the vertical distance between the horizontal line and the top (bottom) of the maxima (minima); an arbitrary distance (which was kept constant for a given peak) was selected for unsymmetrical peaks. The expanded spectrum was usually recorded in approximately 10 min.

The respective hyperfine splittings were easily calculated from the expanded spectrum. $2T_{\parallel}$ was determined from the addition of the four peak separations, and $2T'_{\perp}$ (where $2T'_{\perp} = 2T_{\perp} - 1.6$ gauss) is equal to the separation of peaks 2 and 3 added to the separation of peaks 3 and 4. Several expanded spectra successively measured with the same sample indicated the standard deviation of the S determination was $\pm 0.7\%$. Similarly prepared I(12,3)-labelled liver membrane samples measured on different days indicated a corresponding SD of $\pm 1.2\%$; the larger error is believed to be due in part to daily differences ($\pm 1.0°$) in sample temperatures.

Results

Preparation of Rat Cardiac Sarcolemma

The sucrose density gradient banding pattern found for the cardiac preparations was similar to that previously reported by Kidwai *et al.* [40]. Five 1 ml fractions were assayed for 5′-nucleotidase and protein and examined with phase contrast microscopy. Fig. 2 indicates a representative

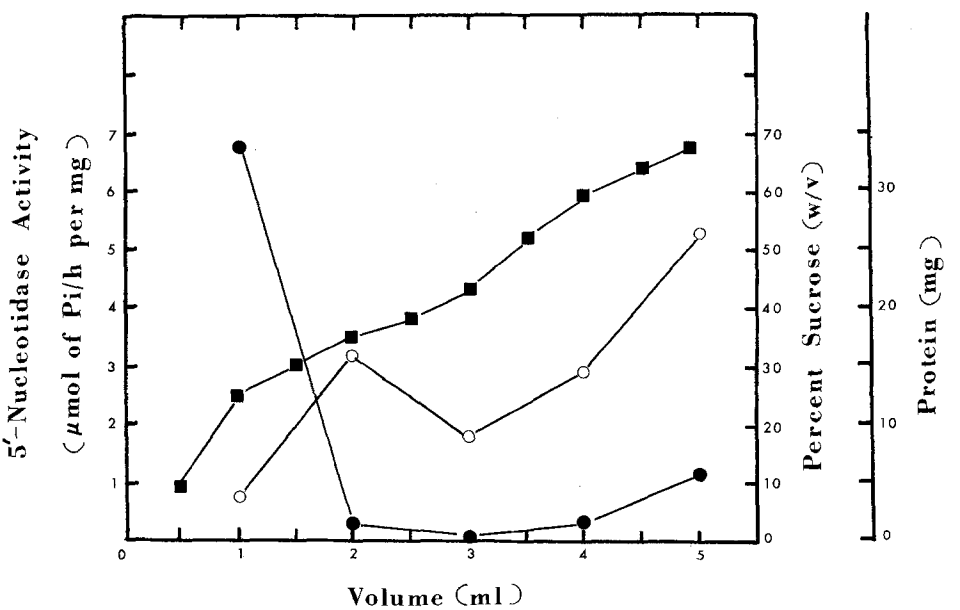


Fig. 2. Profile of filtered and centrifuged rat heart homogenate centrifuged at $100{,}000 \times g$ in a SW 50.1 rotor. (o——o), protein; (●——●), 5′-nucleotidase; (■——■), sucrose concentration. Spin-label studies were performed on membranes collected from fraction 1 (0–1 ml) (*see* Material and Methods)

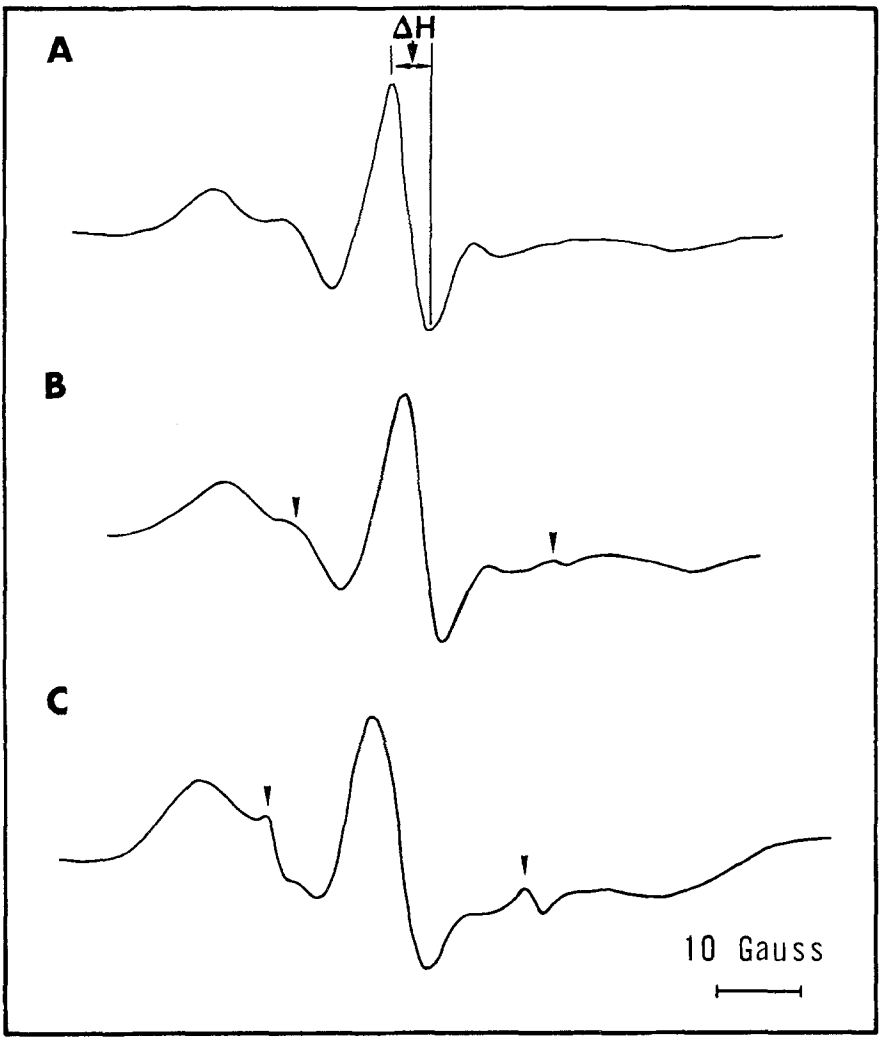
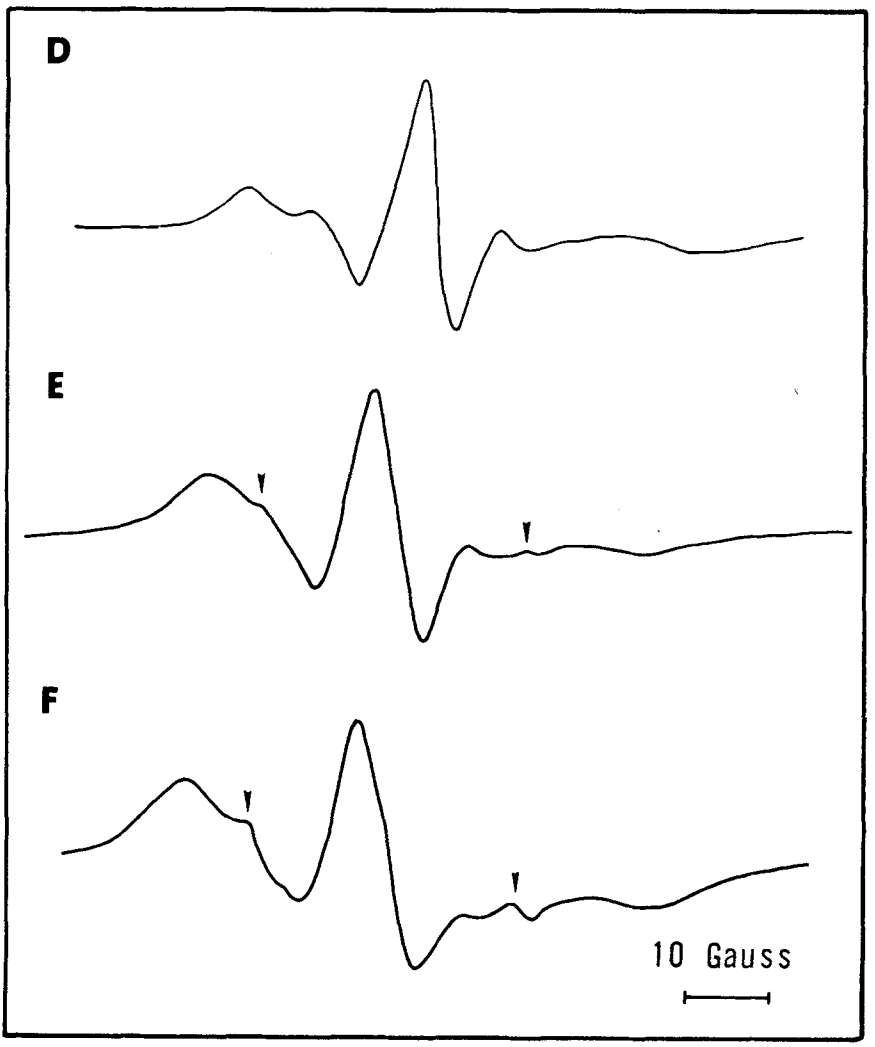


Fig. 3. Unexpanded spectra of rat heart and liver plasma membranes, labelled with "low", "high" and "very high" *I*(12,3) probe concentrations (*see* Table 1). *(A)*, *(B)*, and *(C)* are spectra of heart membranes (22°) with 0.24, 1.44, and 2.88 μg probe/mg protein, respectively; *(D)*, *(E)*, and *(F)* are spectra of liver membranes (31°) labelled with 0.44, 3.37, and 5.4 μg probe/mg protein, respectively. Fig. 3*A* indicates *ΔH* (the peak to peak distance of the central band). The positions of the "fluid" or "liquid-line" spectral components are indicated by arrows in Fig. 3*B*, *C*, *E* and *F*

sucrose density gradient and the distribution of the specific activity of the plasma membrane marker, 5′-nucleotidase. The highest specific activity occurred in the first fraction (which collected at the interface of the loading medium and the sucrose gradient) and is attributed to plasma membrane fragments; the increase in the specific activity over the cor-



responding activity in the filtrate was fivefold. Phase contrast microscopic examinations indicated the presence of membrane fragments in fraction 1 and high concentrations of mitochondria in fraction 2.

I(12,3) Probe Concentration Effects on the ESR Spectra

Rat heart and liver plasma membranes were titrated with varying concentrations of the *I*(12,3) probe and the unexpanded spectra were measured (Fig. 3). The immobilized spectra of spin-labelled rat cardiac

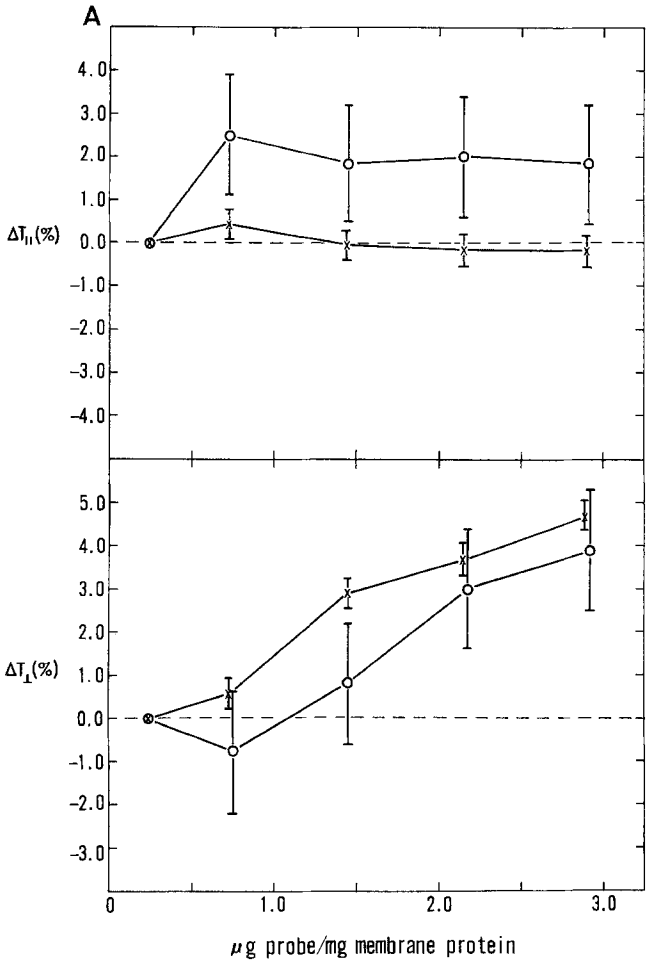


Fig. 4. The $I(12,3)$ probe concentration dependence of the liver (31°) and heart (22°) plasma membrane hyperfine splittings. *(A)* $\Delta T_{\parallel}$ and $\Delta T_{\perp}$, the percent changes in the heart membrane hyperfine splittings from baseline values measured at 0.24 μg probe/mg protein, are plotted as a function of $I(12,3)$ concentration. The control values of $T_{\parallel}$ and $T_{\perp}$ are 27.59 and 9.33 G, respectively. *(B)* $\Delta T_{\parallel}$ and $\Delta T_{\perp}$, the percent changes in the liver membrane hyperfine splittings from baseline values measured at 0.44 μg probe/mg protein, are plotted as a function of $I(12,3)$ concentration. The control values of $T_{\parallel}$ and $T_{\perp}$ are 26.04 and 9.58 G, respectively. The hyperfine splittings were determined from unexpanded (o——o) and expanded (×——×) spectra; the error bars (±1 SD) indicate that the expanded spectra permit a more precise measurement of the splittings

sarcolemma (0.24 μg probe/mg protein) and liver plasma membrane (0.44 μg/mg protein) (Fig. 3*A* and *D*) may be interpreted in terms of a membrane-incorporated $I(12,3)$ molecule undergoing rapid, anisotropic motion about its long molecular axis. A "fluid" or "liquid-line" spectral component appeared in addition to the immobilized spectrum in both

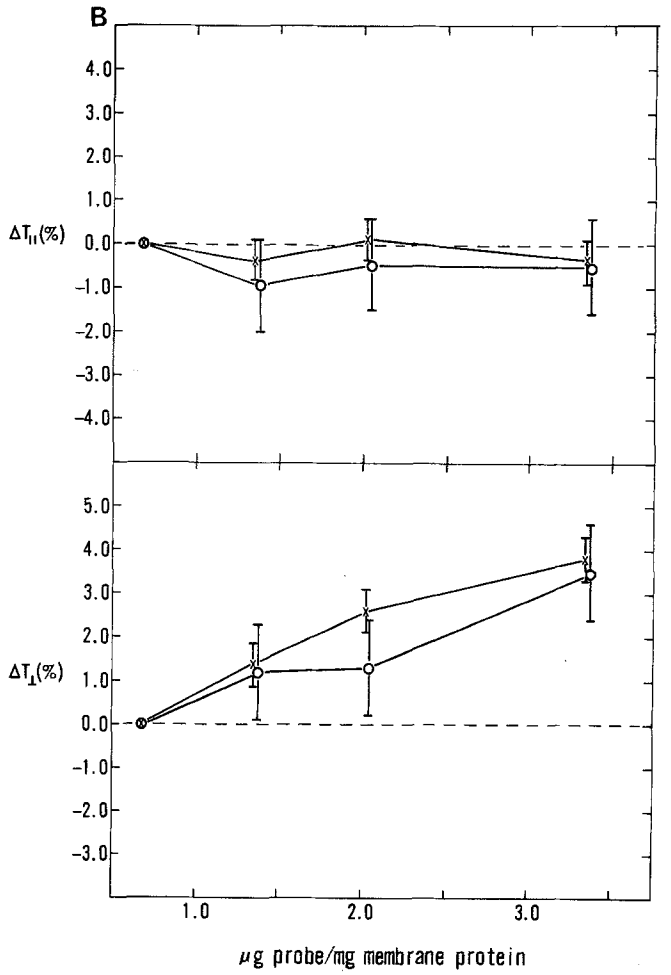


Fig. 4*B*

plasma membrane preparations at higher probe/lipid ratios (Fig. 3*B*, *C*, *E* and *F*).

Additional studies on rat liver plasma membrane (31°) labelled with 4.50 μg *I*(12,3) per mg protein indicated that the probe partitioned between a nonpolar (immobilized) environment and an aqueous (fluid) environment, as judged by the following criteria: (1) the isotropic hyperfine coupling constants for the fluid and immobilized components were 15.58 and 15.07 G, respectively. Here, the a_N value for the fluid component was measured as 1/2 the separation of the "liquid-lines" indicated in Fig. 3*F*, while a'_N for the immobilized component was calculated from Eq. (5); (2) the ESR spectra of membranes probed at high ratios, when centrifuged at 40,000 × *g* and resuspended in fresh buffer, indicated the

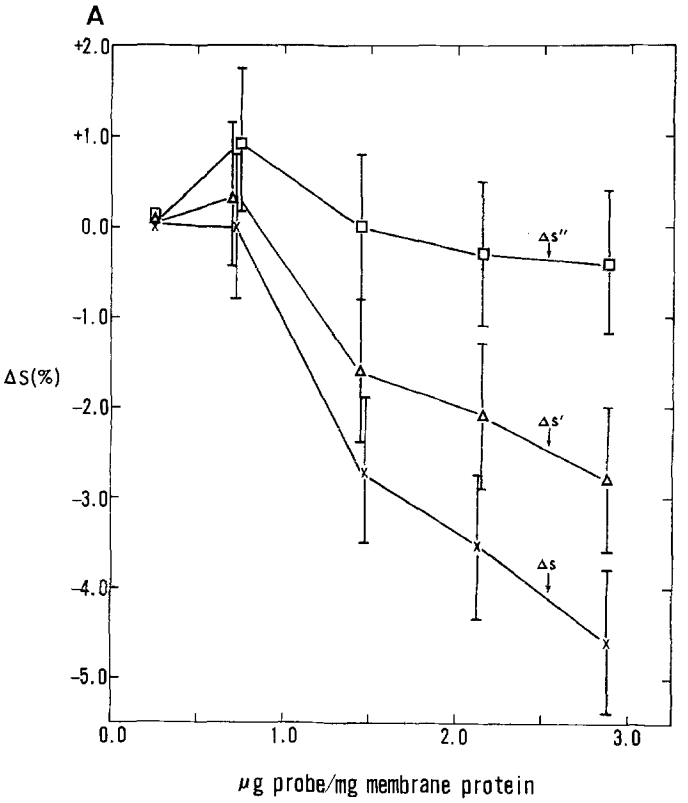


Fig. 5. The effects of increasing $I(12,3)$ probe concentration on the order parameters of heart (22°) and liver (31°) membranes. *(A)* ΔS, $\Delta S'$, and $\Delta S''$, the percent changes in the cardiac sarcolemma order parameters from baseline values measured at 0.24 μg probe/mg protein, are plotted as a function of $I(12,3)$ concentration. The control values of S, S', and S'' are 0.670, 0.695, and 0.726, respectively. *(B)* ΔS, $\Delta S'$, and $\Delta S''$, the percent changes in the liver plasma membrane order parameters from baseline values measured at 0.44 μg probe/mg protein, are plotted as a function of $I(12,3)$ concentration. The control values of S, S' and S'' are 0.618, 0.626, and 0.638, respectively. S[Eq. (8)], S'[Eq. (6)], and S'' [Eq. (7)] were determined from expanded spectra. The error bars indicate ±1 SD

presence of both spectral components; however, the supernatant from the centrifuged membranes yielded spectra containing primarily the isotropic component; and (3) the "fluid" component was preferentially saturated with microwave powers higher than 50 mW [24].

The $I(12,3)$ probe concentration effects on the outer ($T_{\parallel}$) and inner ($T_{\perp}$) hyperfine splittings, calculated from expanded and unexpanded spectra, are plotted as a function of probe concentration for rat heart (22°) and liver (31°) plasma membranes (Fig. 4 *A* and *B*, respectively). The stability of the ESR signal of labelled heart and liver membranes at these temperatures permitted the titration of individual membrane samples with suc-

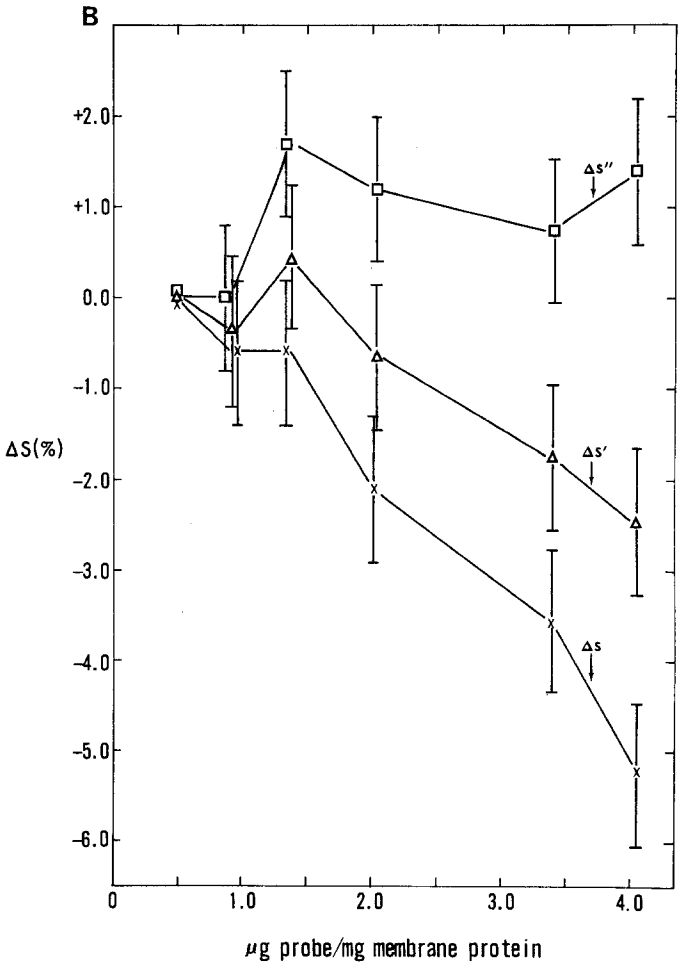


Fig. 5*B*

cessive aliquots of the *I*(12,3) probe; at temperatures exceeding 30°, the signal from labelled heart membranes decreased with time. The $T_{\parallel}$ values were found to be constant for the probe/lipid ratios used to test both membrane systems, while $T_{\perp}$ increased significantly with the probe concentration. The broadening of the inner hyperfine splitting was readily apparent in the expanded spectra, and was also seen in the unexpanded spectra. The higher measurement error using the latter method, however, partially obscured the broadening effect.

The functional dependence of the order parameters *S*, *S′* and *S″* on the *I*(12,3) probe concentration was determined for heart (22°) and liver (31°) plasma membranes (Fig. 5). The *S″* values were independent of the probe concentrations used to examine both membrane systems. However,

Table 1. Characteristic probe concentration ranges of *I*(12,3)-labelled liver and heart membranes

	"Low" μg probe/mg protein ratios	"High" μg probe/mg protein ratios	"Very High" μg probe/mg protein ratios
Membrane systems[a]:			
Rat cardiac sarcolemma (22°)	<0.75	0.75 to 2.50	>2.50
Rat liver plasma membrane (31°)	<1.00	1.00 to 4.00	>4.00
Spectral parameters	ΔS, $\Delta S'$, $\Delta S''$, $\Delta T_{\parallel}$, $\Delta T_{\perp}$, $\Delta a'_N$ and $\Delta m=0$	$\Delta T_{\parallel}$ and $\Delta S''=0$ $\Delta a'_N$, $\Delta T_{\perp}>0$ ΔS, $\Delta S'$ and $\Delta m<0$	$\Delta S''$ and $\Delta T_{\parallel}=0$ (for cardiac sarcolemma and liver plasma membrane) $\Delta S''$ and $\Delta T_{\parallel}>0$ (for erythrocytes [73]) $T_{\perp}$, S, S', a'_N, and m may be difficult to measure
Magnitude of fluid component	Absent Fig. 3 *A* and *D*	Small (or absent) Fig. 3 *B* and *E*	Marked Fig. 3 *C* and *F*

[a] The liver and heart membrane concentrations were 3.3 and 2.3 mg membrane protein per 120 μl buffer.

S and S' decreased substantially if the *I*(12,3) concentration exceeded 0.75 or 1.00 μg/mg protein in heart and liver membranes, respectively; the decrease in S was greater than the corresponding decrease in S'.

Several characteristic probe/lipid ratio ranges may therefore be identified for liver and heart membranes (Table 1): (1) the "low" range was defined for those probe concentrations where the hyperfine splittings, and the order parameters S, S' and S'', were constant (independent of probe concentration). "Intrinsic" membrane properties may therefore be determined from these spectra. A negligible fluid component was also associated with this range (Fig. 3 *A* and *D*); (2) the "high" range was characterized by a broadening of $T_{\perp}$ with increasing probe concentration and the appearance of a small fluid component (Fig. 3 *B* and *E*); $T_{\parallel}$ remained unchanged from the low range values. Therefore, S and S' measured only an "apparent" fluidity in this range, while S'' reflected a polarity-uncorrected, "intrinsic" membrane fluidity; and (3) the "very high" range was identified by noticeably broadened spectra and the presence of a substantial fluid component (Fig. 3 *C* and *F*). Although the inner hyperfine splittings were sometimes difficult to measure, the $T_{\parallel}$ and S'' values were unaffected by these high probe concentrations.

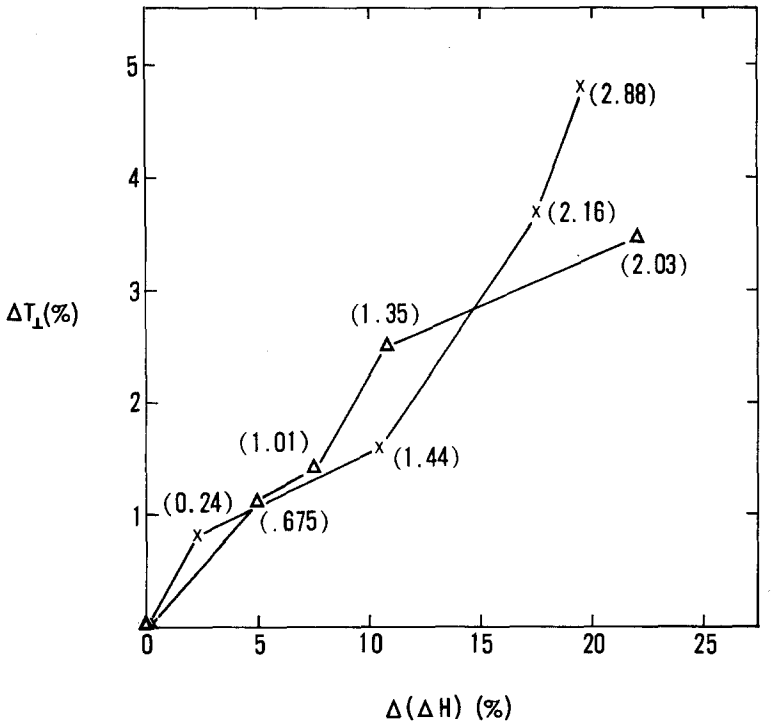


Fig. 6. The percent change in $T_\perp$ ($\Delta T_\perp$) as a function of the percent change in ΔH [$\Delta(\Delta H)$] for heart (×——×) and liver (△——△) membranes labelled with various $I(12,3)$ concentrations. The baseline $T_\perp$ and ΔH values were obtained from heart and liver membranes labelled with 0.15 and 0.44 μg probe/mg protein, respectively. The control $T_\perp$ and ΔH values were 9.33 and 4.3 G for heart membranes, and 9.58 and 4.0 G for liver membranes. $T_\perp$ was measured from expanded spectra, while ΔH was measured from unexpanded spectra. $I(12,3)$ concentrations (μg probe/mg protein) are in parentheses

The "apparent" increase in the fluidity of the liver and heart membranes with probe concentration may be attributed to enhanced radical interactions (i.e., dipole-dipole and spin exchange interactions). For example, the broadening in $T_\perp$ was highly correlated with increases in ΔH (the peak to peak distance of the central band indicated in Fig. 3*A*) (Fig. 6); several investigators have demonstrated that radical interactions can broaden the ΔH of labelled model [13, 14, 60, 61] and biological [62, 64, 73] membranes. The increase in $T_\perp$ also appeared to be associated with such characteristic exchange-broadened effects as the decrease in the high field peak height of the inner hyperfine doublet (peak 4 in Fig. 1*A*) [9, 53, 54] and the downward displacement of the highfield baseline [6, 53, 54, 72].

The *m* ratio [Eq. (9)] is plotted against probe concentration in Fig. 7 for heart (22°) and liver (31°) membranes. The decrease in the *m* ratio in

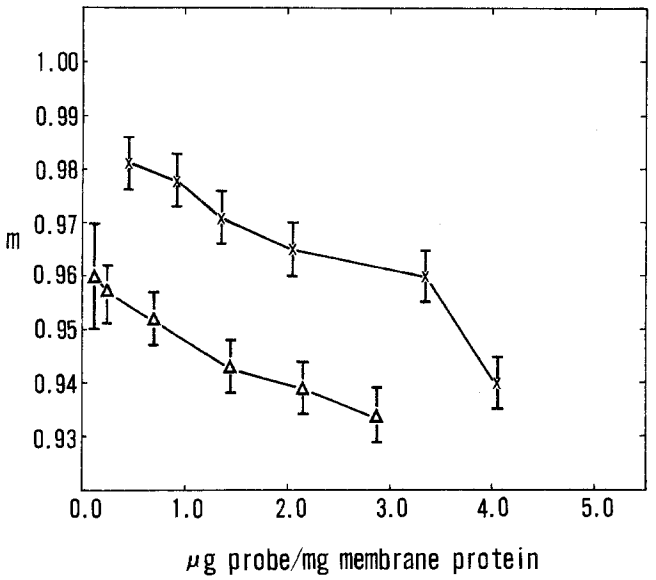


Fig. 7. The $I(12,3)$ probe concentration effects on the m ratio of heart (22°) (△——△) and liver (31°) (×——×) membranes. The m ratios [Eq. (9)] were determined from expanded spectra. The error bars indicate ±1 SD

both membrane systems appears to be due to the enhanced probe-probe interactions which increase $T_\perp$. It is possible to determine the $m°$ values by extrapolating these curves to zero probe concentration: $m° = 0.983$ and 0.960 for liver and heart membranes. Both $m°$ values are less than unity because the polarity of the membrane environment is greater than that of the reference crystal. Heart and liver membrane a'_N values [Eq. (5)] measured at low probe concentrations are 15.41 and 15.06 G, respectively.

Temperature Effects on the Fluidity of Liver Plasma Membrane

The fluidity of $I(12,3)$-labelled rat liver plasma membrane (0.68 μg probe/mg protein) was studied as a function of temperature. Plots of $(1-S)/S$, $(1-S')/S'$ and $(1-S'')/S''$ *vs.* $1/T°\mathrm{K}$ suggested the presence of breaks at 19 and 28° (Fig. 8). The discontinuities were also detected if higher probe concentrations (1.25 and 2.6 μg probe/mg protein) were employed. Fig. 8 was obtained by progressing from low to high temperatures; similar results were observed if spectra were measured by progressing from high to low temperatures.

The $I(12,3)$ probe titration experiments identified 0.68 μg probe/mg protein as a low probe concentration for liver plasma membrane at 31°.

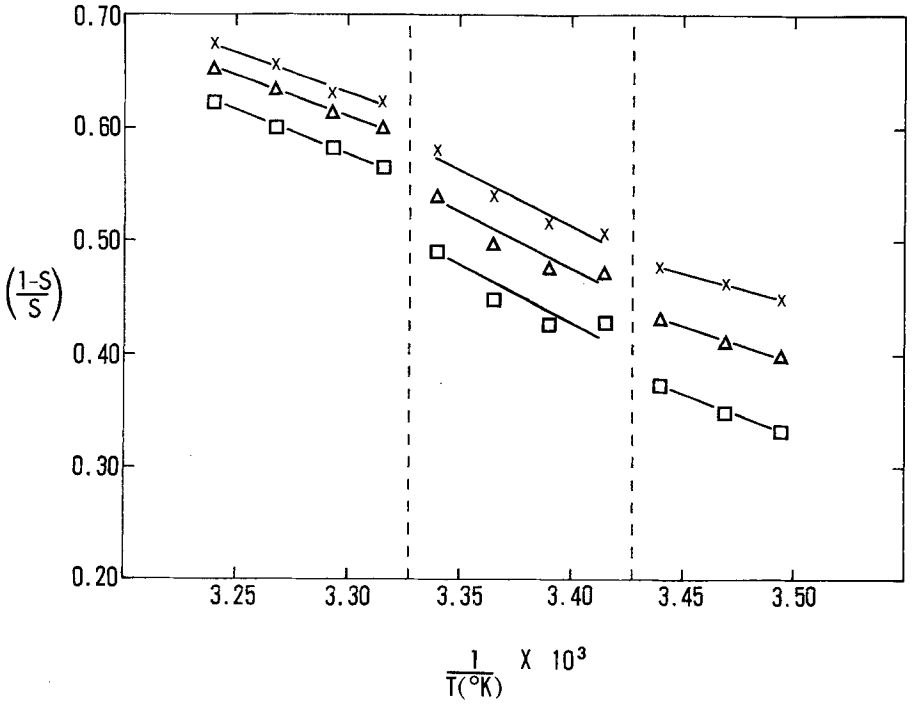


Fig. 8. Temperature dependence of $(1-S)/S$ (×——×), $(1-S')/S'$ (△——△) and $(1-S'')/S''$ (□——□), where S [Eq. (8)], S' [Eq. (6)], and S'' [Eq. (7)] were calculated from expanded spectra of rat liver plasma membrane labelled with 0.68 μg probe/mg protein. The dashed vertical lines indicate discontinuities at 19 and 28°. The temperature range was 11 to 35°

The polarity-corrected S used in Fig. 8 at 31° therefore reflects an "intrinsic" membrane fluidity. It cannot, however, be assumed that liver plasma membrane labelled with this probe concentration will also exhibit negligible radical interactions at lower temperatures. The use of temperatures below 20° has been shown to promote probe-probe interactions in dipalmitoyllecithin labelled with an oxazolidine derivative of androstane [60], and also in sarcoplasmic reticulum probed with a phospholipid spin label [64].

Temperature Effects on the m Ratio of I(12,3)-labelled Liver Membranes

Fig. 9 is a plot of m *vs.* $1/T$°K obtained from liver plasma membrane labelled with a low probe concentration. The discontinuities at 19 and 28° might be caused by abrupt changes in the polarity of the environment of the probe and/or probe-probe interactions. It is not yet possible

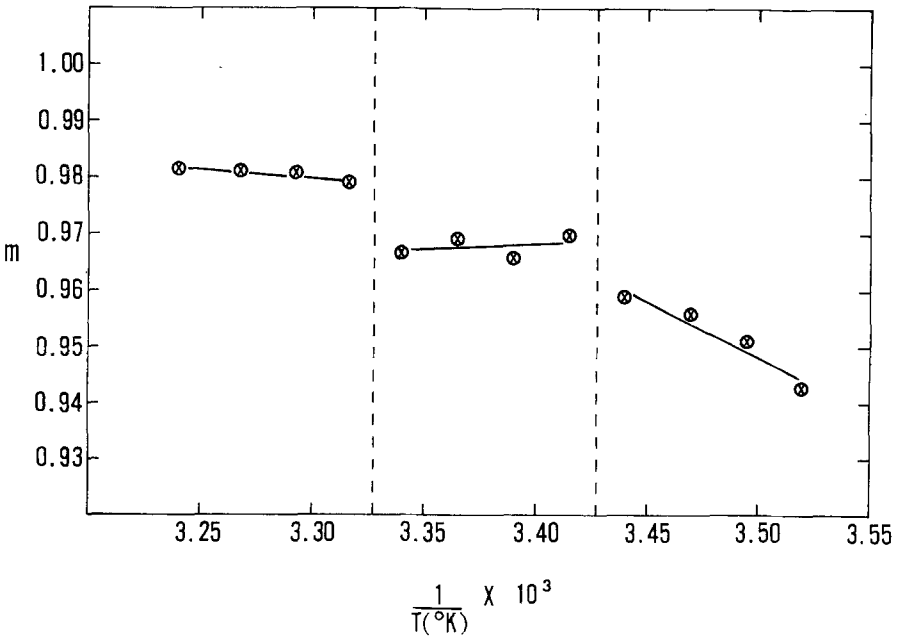


Fig. 9. Temperature dependence of the *m* ratio, *m* [Eq. (9)] calculated from expanded spectra of rat liver plasma membrane labelled with 0.68 μg probe/mg protein. The dashed vertical lines indicate discontinuities at 19 and 28°. The temperature range was 11 to 35°

to unequivocally assess the importance of probe-probe interactions in this plot. Additional studies of *m* as a function of temperature using higher (and lower) probe concentrations may help to resolve this uncertainty. Lastly, the *m* ratio of rat liver plasma membrane appears to be relatively insensitive to temperature; the variation of *m* for the range 20–35° is 0.0007/°C.

Microwave Power Effects on Liver Membrane Spectra

The nonresonant absorption of microwaves by water may heat aqueous membrane samples above ambient temperature. Microwave heating may be estimated for *I*(12,3)-labelled membranes by studying the ESR spectra as a function of microwave power; for example, an increase in sample temperature should decrease the calculated order parameter. Table 2 indicates that the spectra of *I*(12,3)-labelled liver membranes were not significantly perturbed at the microwave power used in this study (40 mW). It should also be noted that the *m* ratio was unaffected by high microwave powers. The ESR spectra of the immobilized component were not noticeably saturated at 40 mW.

Table 2. Microwave power effects on various spectral parameters[a] of *I*(12,3) labelled rat liver plasma membrane[b]

Microwave power (mW)	$T_{\parallel}$(G)	$T_{\perp}$(G)	S	ΔS(%)	m
5	26.07	9.75	0.608	–	0.971
10	25.93	9.70	0.607	0.0	0.978
32	25.86	9.74	0.604	−0.6	0.978
50	25.83	9.73	0.603	−0.7	0.979
100	25.74	9.75	0.600	−1.3	0.980

[a] Spectra were recorded at 31°. The hyperfine splittings, $T_{\parallel}$ and $T_{\perp}$, were measured from expanded spectra as indicated in Fig. 1; the 0.8 gauss addition to the apparent $T_{\perp}$ splitting is included. S and m were calculated from Eqs. (8) and (9), respectively; ΔS is the percent change in the order parameter from the value calculated using 5 mW microwave power.
[b] Membrane labelled with 0.680 μg *I*(12,3) per mg protein.

Table 3. Effects of $CaCl_2$ on various spectral parameters[a] of *I*(12,3) labelled rat liver plasma membrane[b]

$CaCl_2$ (mM)	$T_{\parallel}$(G)	$T_{\perp}$(G)	S	S'	S''	m
(31°)						
0.0	25.73	9.70	0.602	0.609	0.619	0.984
1.3	26.04	9.58	0.618	0.626	0.637	0.983
(37°)						
0.0	24.63	9.98	0.558	0.557	0.557	1.000
2.0	24.88	9.88	0.570	0.571	0.571	0.999
3.8	25.51	9.75	0.594	0.599	0.607	0.987
5.5	25.59	9.76	0.595	0.602	0.612	0.984
7.0	25.39	9.68	0.596	0.597	0.600	0.996

[a] The hyperfine splittings, $T_{\parallel}$ and $T_{\perp}$, were measured from expanded spectra as indicated in Fig. 1; the 0.8 gauss addition to the apparent $T_{\perp}$ splitting is included. S, S', S'' and m were calculated as indicated in the text.
[b] Membrane labelled with 0.680 μg *I*(12,3) per mg protein.

$CaCl_2$ Effects on the Spectra of Liver Plasma Membrane

The effects of calcium on the fluidity of rat liver plasma membrane (labelled with a low probe concentration) have been investigated at 31 and 37°. Table 3 shows that $CaCl_2$ additions at these temperatures significantly increased (decreased) the outer (inner) hyperfine splittings. The fluidity of the membrane was therefore decreased by calcium, as indicated by positive increases in S, S' and S''. Calcium does not appear to enhance probe-probe interactions in liver membranes at these temperatures since no changes were noted in the m ratio upon addition of $CaCl_2$.

Discussion

The interpretation of ESR spectra of spin-labelled membranes in terms of the structure of the host matrix is not necessarily straightforward. The membrane-incorporated nitroxide probe represents a perturbation whose magnitude is often difficult to assess. Keith and co-workers have demonstrated that spin labels tumble freely in a variety of lipids at temperatures below their respective bulk melting points [50]. The mobility of the spin probe in such cases appears to reflect a fluid environment whether dissolved in bulk liquid or solid. Furthermore, incubation of intact erythrocytes with $I(12,3)$ has been shown to induce echinocyte morphology and cell lysis at nitroxide stearate concentrations of 10^{-8} and 10^{-5} M, respectively [4].

Another frequent criticism of the spin-label technique is the uncertainty as to whether the probe detects average or local membrane properties. A spin probe which is uniformly distributed throughout the membrane should represent the fluid and crystalline phases of mixed systems in proportion to their relative concentrations. However, $I(m, n)$ probes have been found in several binary phase, model lipid systems to partition preferentially into the more fluid phase [6, 56, 55]. Moreover, Wallach and co-workers interpreted the $I(m, n)$-induced perturbation of the erythrocyte membrane in terms of a segregation of the probe in the membrane [4]. The presence of high local concentrations of probe was confirmed from the noticeably exchange-broadened spectra [73]. Probe-probe interactions such as these may obscure the nature of "intrinsic" membrane properties. Here, intrinsic membrane properties refer to those which are measured when radical interactions (e.g., dipole-dipole and electron-electron exchange) are negligible.

Spin probes have proven to be valuable investigative tools for studies of membrane structure and function despite these inherent uncertainties. Although the motion of a spin label may not reflect the corresponding motions of native lipid molecules in the membrane, the nitroxide derivative is nevertheless a sensitive reporter of changes in its local environment. The possibility that a given label samples only the more fluid regions of the membrane may be evaluated through the use of labels with widely different structures. Lastly, the question as to whether intrinsic or apparent membrane properties are being measured may be answered by conducting careful probe titration experiments.

A $I(12,3)$ probe concentration-dependent increase in $T_{\perp}$ (but not $T_{\parallel}$) has been observed in the expanded and unexpanded spectra of rat liver

and heart plasma membranes (Fig. 4). The hyperfine splittings may be measured with greater precision if expanded spectra are used. The precision of the order parameter, S, calculated from expanded spectra, was estimated to be $\pm 0.7\,\%$; this error is comparable to that obtained in a $I(12,3)$ lymphocyte study which used the Nicolet 1020A signal averager [15]. It is suggested that the expansion technique may prove useful in future $I(m, n)$-membrane studies.

The broadening of the inner hyperfine splitting in the high and very high ranges may be explained by a corresponding increase in probe-probe interactions. The spectral broadening was progressively more significant as the probe concentration increased from the low to high to very high ranges (Fig. 3). However, the broadening in Fig. 3 *B* and *E* might be overlooked if only these spectra were considered, since no single feature is unequivocally characteristic of enhanced probe-probe interactions.

It is important to consider how successfully the various order parameters S, S' and S'' describe membrane fluidity within the context of these probe concentration effects. S'', being a function of only $T_{\|}$, might appear to be the most useful since it was constant for those probe concentrations used to examine heart and liver membranes. However, S'' is a function of both the polarity and fluidity of the membrane; changes in S'' necessarily reflect fluidity alterations only in the limiting case where the polarity of the membrane is identical to that of the reference crystal. Thus, previous $I(m, n)$ label studies which interpreted changes in S'' (or $T_{\|}$) only in terms of a membrane fluidity change overlooked the possible involvement of polarity alterations [11, 17, 21, 35, 43, 57–59, 66]. Nor can it be assumed that $T_{\|}$ will be insensitive to probe-probe interactions in all membrane systems, since increases in $T_{\|}$ with probe concentration were noted in a $I(12,3)$-labelled erythrocyte membrane study [73]. S' is generally less useful than S'' in the description of liver and heart membrane fluidities because it not only depends on membrane polarity but is also sensitive to the probe concentration in the high and very high ranges.

The polarity-corrected order parameter (S) affords the most accurate description of the membrane fluidity, if experimentally determined low probe concentrations are used. However, S is very sensitive to the probe concentration since the polarity correction includes a multiply-weighted $T_{\perp}$ term [*see* Eq. (8) and Fig. 5]. Thus, the apparent fluidity determined for heart and liver membranes at high probe concentrations was found to be overestimated by as much as $5\,\%$. These results are consistent with Hubbell and McConnell's observation that the order parameter S of

I(10,3)-labelled dipalmitoyllecithin decreased as the probe/lipid ratio increased from 1:600 to 1:100 [29].

Thus, the use of "high" probe concentrations such that radical interactions are present may prevent the measurement of "intrinsic" membrane properties. These findings raise several important questions. What probe concentrations constitute low probe/lipid ratios in a given membrane system? Are the steps usually taken to minimize probe-probe interactions sufficient to insure that intrinsic properties will be measured? Lastly, what qualitative or quantitative "markers" might indicate that high probe concentrations are being used?

Probe-probe interactions are frequently assumed to be minimal in spin-label membrane studies if low calculated probe/lipid ratios (e.g., less than 1:100) are employed. Although the above assumption is justified if the label is distributed uniformly in the bilayer, recent investigations of model and biological membranes raise the possibility that various spin probes segregate in local membrane patches. Thus, the "effective" probe concentrations in the local lipid domains may be quite high even if a low probe/*total lipid* ratio is used. The probe concentration at which radical interactions are negligible can be determined for a given membrane system only by conducting careful titration experiments.

It is also sometimes assumed that a characteristic exchange broadened spectrum will identify the presence of significant probe-probe interactions. The "very high" range spectra of rat liver and heart membranes certainly exhibit markedly broadened features. However, the probe-probe interactions present in the "high" range spectra (Fig. 3*B* and *E*) can be unequivocally assigned only by referring to low range spectra. For example, the decrease in the high field peak height of the inner hyperfine doublet (identified in Fig. 1*A*) below the baseline might be incorrectly attributed to a slower motion of the probe (i.e., less rapid averaging of the g_{xx} and g_{yy} tensor elements) [9, 33, 48] in the absence of studies at lower probe concentrations. Therefore, the resemblance of the "high" range spectra obtained here (Fig. 3*B* and *E*) to the corresponding spectra of other membrane systems labelled with presumably low probe concentrations [7, 8, 11, 17, 25, 26, 34, 36, 43, 44, 52, 58, 59, 62, 65, 66, 70] suggests the possibility that radical interactions might have interfered with the measurement of intrinsic membrane properties in previous studies.

The presence of a fluid component superposed over the immobilized spectrum was noted in rat liver and heart plasma membranes labelled with high and very high probe concentrations. Similar fluid components have been observed for a wide variety of *I*(*m*, *n*)-labelled model and biological membrane systems, including erythrocytes [36, 44, 73], influenza virus [43],

normal and transformed 3 T3 cells [24], egg lecithin and dipalmitoyllecithin liposomes [6], spheroplasts from *Bacillus stearothermophilus* [21], guinea pig ileum plasma membrane [65], lymphocytes and L cells [36]. Our experiments on rat liver membranes suggest that the probe partitions between the membrane lipid and the aqueous environment, in agreement with similar studies performed on erythrocytes [8] and model membrane liposomes [6]. At the liver and heart membrane concentrations employed in this study, the onset of probe-probe interactions was correlated with the appearance of a fluid component in liver and heart membranes. This suggests the possibility that previous $I(m, n)$ membrane studies with fluid components may also have employed "high" probe concentrations. However, the presence of a substantial fluid component is not an unequivocal marker of high probe concentrations, since the magnitude of the fluid component also depends on the membrane concentration and the lipid-water partition coefficient of the probe.

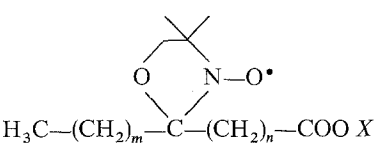
An uncertainty as to whether low probe/lipid ratios were used can be definitively resolved only by examining the dependence of the hyperfine splittings on probe concentration. However, the m ratios [Eq. (9)] may be of use in the quantitative assessment of probe-probe interactions if titration data is unavailable, since:

1. The m ratio is sensitive to radical interaction broadening of either $T_{\parallel}$ or $T_{\perp}$, since increases in either $T_{\parallel}$ or $T_{\perp}$ will decrease m.

2. The m ratio of $I(12, 3)$-labelled rat liver plasma membrane was found to be relatively independent of both temperature alterations in the range 20–35° and microwave heating at 31°.

3. Radical interaction effects will solely be responsible for deviations in the m ratio from unity when the polarity of the membrane is identical to that of the reference crystal [i.e., Trace (**T**) = Trace (**T**′)].

An examination of the m ratios obtained from various membranes labelled with a particular class of spin probes may therefore be of interest. Table 4 gives the spectra measurement conditions for a number of unoriented model and biological membranes probed with spin labels having the general formula:



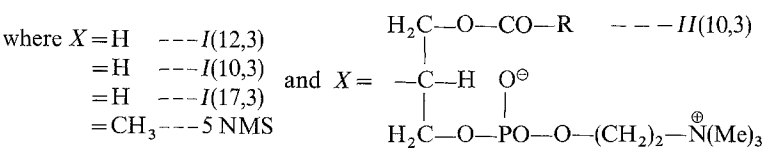


Table 4.

Membrane System	No. in Fig. 10	Spin Label	Treatment	Buffer	$T°$
Egg lecithin (2%) [2]	1		pH = 4.04	NaAc/HAc, sodium PO_4,	*RT*
	3		pH = 4.50	or Tris-HCl (ionic	
	4		pH = 5.00	strength 0.05 M)	
	5	10^{-4} M	pH = 5.46		
	7	*I*(12,3)	pH = 5.92		
	14		pH = 6.48		
	20		pH = 6.92		
	23		pH = 7.40		
Sarcoplasmic vesicles (Rabbit), 20 mg protein per ml [68]	32	1.25 mg *I*(12,3) per 100 mg protein	–	0.1 M KCl	*RT*
Dispersion of oleic acid (2.8 mg/ml) and poly -*L*-lysine (6 mg/ml) [68]	38	*I*(12,3)			
Oleic acid dispersion (10 mg/ml)	6			pH 8.5, 0.1 M KCl	
Egg yolk lecithin (20 mg per ml) [68]	19			0.1 M Tris-HCl, pH 8.5	
Myotonic human erythrocytes [7]	21	5 NMS	–	0.15 M NaCl,	20
Normal human erythrocytes [7]	44			10 mM Tris-HCl, pH 7.4	
Rabbit erythrocyte lipid vesicles (6.2 mg lipid/ml) [26]	51	1:100 mole ratio *I*(12,3)	–	NaCl-KCl (0.145 M),	*RT*
	53		20 μg mellitin per 50 μl	pH 7.2	
	54		50 μg mellitin per 50 μl		
Rabbit erythrocyte ghosts (6.2 mg lipid/ml) [26]	58	*I*(12,3)	–	0.05 M phosphate	
	64		50 μg mellitin per 50 μl	buffered saline, pH 7.2	
	59		60 μg mellitin per 50 μl		

Rabbit erythrocytes (6.2 mg lipid/ml) [26]	52	*I*(12,3)	–	0.05 M phosphate buffered saline, pH 7.2	*RT*
	57		0.01 % Triton *X*-100		
	56		0.025 % Triton *X*-100		
	47		0.05 % Triton *X*-100		
	50		0.075 % Triton *X*-100		
	49		0.10 % Triton *X*-100		
	11		0.50 % Triton *X*-100		
	8		1.00 % Triton *X*-100		
	10		2.00 % Triton *X*-100		
Liquid crystalline beef heart cardiolipin [25]	12	20 µg *I*(12,3) per mg lipid	–	0.145 M NaCl-KCl, 50 mM Tris-HCl, pH 7.0	20
	60		0.05 M $CaCl_2$		
Halobacterium cutirubrum membrane vesicles [20]	62	1:200 *I*(12,3) to lipid molar ratio	–	3.4 M NaCl, 0.05 M Hepes, $MgCl_2 \cdot 6H_2O$ 20 g/liter, $CaCl_2 \cdot 2H_2O$ 0.2 g/liter, pH 7.0	22
	35				37
	61		Heat denaturation		22
	34				37
Halobacterium cutirubrum lipid extracts [20]	43		–		22
	13				37
Halobacterium cutirubrum membrane vesicles [20]	55		–	$MgCl_2 \cdot 6H_2O$ 20 g/liter, KCl 2 g/liter, $CaCl_2 \cdot 2H_2O$ 0.2 g/liter	22
	33				37
Rat liver plasma membrane	28	0.68 µg *I*(12,3) per mg protein	–	5 mM Tris-HCl, 8 % (w/v) sucrose, pH 7.6	31
	25	1.35 µg *I*(12,3) per mg protein			
	27	2.03 µg *I*(12,3) per mg protein			
	24	3.37 µg *I*(12,3) per mg protein			
Rat cardiac sarcolemma	42	0.24 µg *I*(12,3) per mg protein	–	5 mM Tris-HCl, 8 % (w/v) sucrose, pH 7.6	22
	45	0.72 µg *I*(12,3) per mg protein			
	41	1.44 µg *I*(12,3) per mg protein			
	40	2.16 µg *I*(12,3) per mg protein			
	39	2.88 µg *I*(12,3) per mg protein			

Table 4 (continued)

Membrane System	No in Fig. 10	Spin Label	Treatment	Buffer	T^0
Sonicated soybean phosphatide suspension (Asolectin) [28]	26	*I*(17,3)	–	0.05 M Tris-HCl, pH 8.0	*RT*
Ca^{2+}-beef heart cardiolipin complex (hexagonal) [5]	15	1:150 *I*(10,3) to lipid molar ratio	75 mM $CaCl_2$	20 mM phosphate, 0.15 M NaCl, pH 7.2	*RT*
Na^+-beef heart cardiolipin (lamellar) [5]	16		–		
Egg lecithin [5]	18				
Pig liver monoglycerides [5]	9				
Bovine brain gangliosides (micellar) [5]	2				44
Bovine brain gangliosides: cholesterol 1:1 [5]	22				
Human erythrocytes [36]	63	*I*(12,3)	–	Phosphate buffered saline	23
Human lymphocytes [36]	31				
Mouse L-cells [36]	37			Eagle's minimal essential medium	
3T3 mouse fibroblasts (whole) [24]	30	*I*(10,3)	–	Phosphate buffered saline, pH 7.3	25
	17				37
Transformed 3T3 mouse fibroblasts (whole) [24]	29				37
Normal human erythrocytes [8]	48	5 NMS	–	Isotonic Tris-HCl (0.01 M), pH 7.4	*RT*
Myotonic human erythrocytes [8]	36				
Egg lecithin/cholesterol 2:1 molar ratio [29]	46	*II*(10,3)	–	Distilled H_2O	–

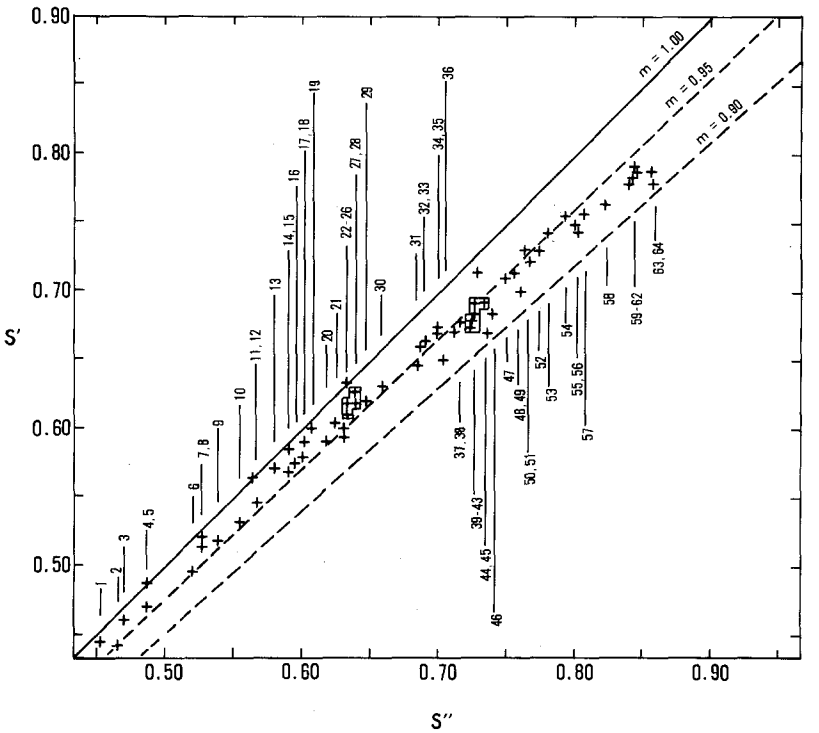


Fig. 10. A S' *vs.* S'' (or m) plot obtained from spin label studies on unoriented model and biological membranes. The m lines corresponding to 1.00, 0.95, and 0.90 are indicated. Data from rat liver (31°) and heart (22°) membranes labelled with various $I(12,3)$ concentrations (Table 4) are enclosed in rectangles lying along $S''=0.64$ and 0.73, respectively; the hyperfine splittings, $T_{\parallel}$ and $T_{\perp}$, were measured from expanded spectra (*see* Fig. 1 *B* and Material and Methods). Data are also included from previous labelled membrane studies and are listed corresponding to the numbering in Table 4; the hyperfine splittings, $T_{\parallel}$ and $T_{\perp}$, were measured from unexpanded spectra as in Fig. 1 *A*. S' and S'' values were calculated from Eqs. (6) and (7), respectively. The 0.8 G correction [29] was added to the $T_{\perp}$ splitting, if not already included. $(T_{xx}, T_{yy}, T_{zz})=(6.1, 6.1, 32.4)$ G [67]

A plot of S' *vs.* S'', determined from the labelled membrane systems in Table 4, is shown in Fig. 10. The m ratios in this figure were found to be either equal to or less than one. A decrease in m from unity may be (1) due to the probe sampling a membrane environment which is more polar than that of the reference crystal; and/or (2) the result of radical interaction broadening of $T_{\parallel}$ or $T_{\perp}$. However, the individual contributions to the lowering of m is (in general) difficult to assess. The m ratios of rat liver (31°) and heart (22°) membranes determined at various probe concentrations are enclosed in rectangles lying along $S''=0.64$ and 0.73, respectively; the m ratios calculated at low (high) probe/lipid ratios lie closest to (furthest from) $m=1.0$. It is of particular interest to note that the apparent

Table 5. $I(m, n)$ probe studies of egg lecithin and erythrocyte membranes at room temperature[a]

Membrane system	Spin label	pH	Buffer	S	S'	S''	m
Egg lecithin (2%) [2]	10^{-4} M $I(12,3)$	6.92 7.40 8.50	Tris-HCl (ionic strength 0.05)	0.57 0.58 0.58	0.59 0.60 0.60	0.62 0.63 0.63	0.95 0.96 0.96
Egg lecithin [5]	1:150 $I(10,3)$ to lipid molar ratio	7.2	20 mM phosphate 0.15 M NaCl	0.58	0.59	0.60	0.98
Egg lecithin (20 mg/ml) [68]	$I(12,3)$	8.5	0.1 M KCl	0.60	0.60	0.61	0.99
Human erythrocytes [36]	$I(12,3)$	–	Phosphate buffered saline	0.71	0.78	0.86	0.91
Rabbit erythrocyte ghosts [26]	$I(12,3)$	7.2	Phosphate buffered saline (0.05 M)	0.72	0.76	0.82	0.93
Rabbit erythrocytes				0.70	0.73	0.77	0.95

[a] The hyperfine splittings were measured from spectra of unoriented membranes as in Fig. 1*A*; the 0.8 G correction [29] was added to the $T_\perp$ splitting, if not already included. The m values and the order parameters S, S' and S'' were calculated as indicated in the text, with $(T_{xx}, T_{yy}, T_{zz})=(6.1, 6.1, 32.4)$ G [67]. A small "fluid" component was reported only for the human erythrocyte spectrum [36].

order parameters of liver and heart membranes measured at low m ratios are lower than their corresponding intrinsic order parameters by 5.2 and 4.6%, respectively.

The m ratio may prove to be a useful parameter in comparing those $I(m, n)$-labelled membrane systems measured under similar conditions. For example, several $I(m, n)$ studies have been conducted on egg lecithin dispersions in comparable buffers at room temperature. An examination of the effects of pH on $I(12, 3)$-labelled egg lecithin indicated that the fluidity ($S=0.58$) and m ratio ($m=0.96$) were constant in the pH range 7.4 to 8.5 [2] (Table 5). However, this m ratio is considerably lower than the corresponding values obtained in two other $I(m, n)$ egg lecithin studies (Table 5). The difference in the m ratios might be attributed to the use of relatively "high" probe concentrations in the Barratt and Laggner study [2].

The m ratios of $I(12, 3)$-labelled human and rabbit erythrocytes (RT) also exhibit large differences (Table 5). It is tempting to speculate that this scatter was due to the use of different probe/lipid ratios since $T_{\|}$ has been found to be sensitive to probe concentration in a human erythrocyte

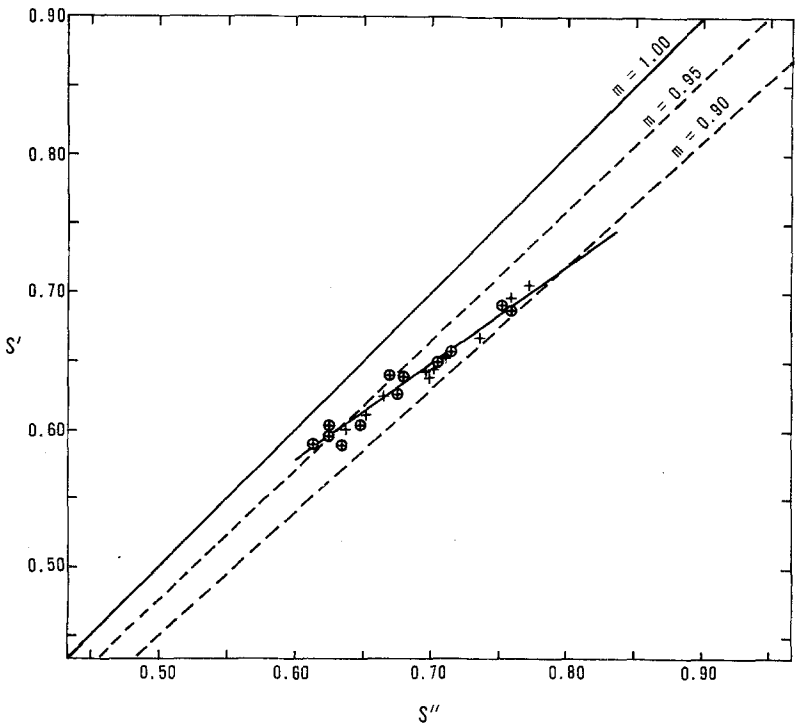


Fig. 11. A S' *vs.* S'' (or m) plot obtained from a 5 NMS (5-nitroxide methyl stearate) study at 20° of normal and myotonic erythrocyte membranes suspended in 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4) [7]. The hyperfine splittings used to calculate S' [Eq. (6)] and S'' [Eq. (7)] were measured from unexpanded spectra as in Fig. 1*A*; the 0.8 G addition to $T_{\perp}$ was included. The m values for normal (+) and myotonic (⊕) erythrocyte membranes decrease as S'' increases; a least squares line, determined for the normal and diseased membrane S' and S'' values, verifies this effect. The coefficient of determination of the least squares fit (r^2) is equal to 0.97. The order parameters were calculated using $(T_{xx}, T_{yy}, T_{zz}) = (6.1, 6.1, 32.4)$ G [67]

membrane study [73]. The above interpretation is nevertheless subject to considerable criticism. Human and rabbit erythrocytes may exhibit a species-dependent difference in the membrane polarity which would invalidate any m ratio comparison. Differences in the preparation of the erythrocyte membranes and the conditions used to record the spectra (e.g., buffer and temperature) might also cause the m ratio to vary.

The above membrane m ratio comparisons therefore suggest, but do not prove, that high probe concentrations were used in previous $I(m, n)$ studies. A more critical comparison can be made of the data from a 5 NMS (5-nitroxide methyl stearate) study of normal and myotonic muscular dystrophic erythrocytes in which spectra were measured with separate samples employing the same temperature and buffer [7]. Fig. 11 is a S' *vs.* S'' plot obtained from the labelled normal and myotonic mem-

brane samples. The S'' and m values of each membrane system varied considerably, although all spectra were measured with the same buffer at 20°. Moreover, Fig. 11 shows that the m ratios generally decreased with increasing S'' values. Both these effects could be attributed to the use of a wide range of probe concentration, if either $T_{\parallel}$ or $T_{\perp}$ depended on probe concentration. The lowered high field peak height of the inner hyperfine doublet in the spectrum of 5 NMS normal human erythrocytes is also consistent with this interpretation [7]. The use of "high" probe concentrations might also be inferred for those $I(12, 3)$ erythrocyte studies in Fig. 10 and Table 4 which share low m and high S'' values.

The discontinuities noted at 19 and 28° in the temperature dependence of the fluidity of $I(12, 3)$-labelled liver plasma membrane may be explained in terms of a phase separation [10, 46, 47, 54] occurring between these temperatures. The lower and higher temperature breaks might correspond to the beginning and end of the melting of the lipid hydrocarbon chains. Both discontinuities were readily detected from an examination of expanded spectra. However, only the 19° break was found in an earlier study which measured hyperfine splittings from unexpanded spectra (Fig. 2 in [63]); the presence of the 28° discontinuity was partially obscured due to the larger error in the S measurement. It is of interest to compare these liver plasma membrane results with a temperature dependence study of the fluidity of 6N11 (an oxazolidine derivative of eidecane) labelled liver microsomes, which indicated breaks at 19 and 32° [19].

It is possible that the phase separation detected in liver plasma membrane involves local lipid regions. The presence of probe-probe interactions at relatively low probe/phospholipid ratios (1:258) suggests that the probe may not be uniformly distributed throughout the liver membrane. The lipid domains recently observed in liver plasma membrane with selected dark-field electronmicroscopy [32] may not share the same fluidity, and the $I(12, 3)$ probe may primarily reside in the more fluid regions. Such an interpretation would be consistent with previous reports that $I(m, n)$ probes in mixed binary phases preferentially sample the more fluid phase [6, 55, 56].

These temperature studies support the contention that phospholipids play an important role in the hormonal stimulation of adenylyl cyclase [12, 74]. Kreiner and co-workers have detected an abrupt increase at 32° in the activity of hepatic adenylyl cyclase in the presence of glucagon or epinephrine [37, 41]; the basal activity of adenylyl cyclase did not exhibit such a change at this temperature. It is possible that the phase separation observed in liver plasma membrane suspended in a Tris-sucrose buffer is

strongly correlated with the increase in activity (at 32°) of hormone-stimulated adenylyl cyclase, determined in a magnesium-containing buffer. If it is true that the $I(12, 3)$ probe primarily samples fluid lipid regions, it is tempting to infer that some feature of the hormone receptor-transducer-effector complex may also be associated with the more fluid phase.

The addition of various calcium ion concentrations apparently increases the rigidity of rat liver plasma membrane labelled with "low" $I(12, 3)$ probe concentrations. Calcium has also been reported to mediate decreases in the fluidity of several other $I(12, 3)$-labelled membranes, including *Bacillus subtilis* cytoplasmic membranes [17] and the model membranes phosphatidylserine [54] and cardiolipin [25]. However, these findings must be regarded with caution since calcium has been shown to induce probe segregation in several model membranes [6, 53, 54]. It is necessary to establish (if possible) the role that alterations in radical interactions played in each of the above membrane systems. Probe-probe interactions were judged to be negligible in the unexpanded spectra of $I(12, 3)$-labelled rat liver plasma membrane and phosphatidylserine before and after addition of calcium; moreover, the m ratio of liver plasma membrane was not changed by calcium binding at 31 and 37°. Both the *Bacillus subtilis* [17] and cardiolipin [25] studies neglected the possibility of a divalent cation-mediated probe segregation in the bilayer. However, the unexpanded spectrum of $I(12, 3)$-labelled cardiolipin obtained after addition of $CaCl_2$ appears to exhibit characteristic exchange-broadened features. The decrease of the m ratio of cardiolipin from 1.00 to 0.93 also argues for an increase in radical interactions which might have interfered with the detection of membrane structural changes. These studies illustrate the difficulties involved in interpreting agent-perturbation studies using labelled membranes.

Conclusions

The above findings seem to illustrate some fairly general principles which should be followed in $I(m, n)$ membrane studies.

I. The comparison of "intrinsic" properties of membrane systems at a fixed temperature requires the use of experimentally determined "low" probe/lipid ratios. The following protocol is suggested to ascertain what probe/lipid ratios are appropriate for a given membrane system:

A. A sufficient quantity of $I(m, n)$ probe should be incorporated into the membrane to permit the recording of an ESR trace with a reasonable signal to noise level. The presence of the following "markers" might suggest the use of "high" probe concentrations: (1) a value of m less than 0.95; (2) a substantial "fluid" component; and/or (3) the height of the high field peak of the inner hyperfine doublet (peak 4 in Fig. 1*A*) being below the baseline. Spectra which share the last feature must be viewed with considerable caution, since the order parameter formalism may not be applicable [9] even if radical interactions are absent.

B. The effects of wide ranges of probe concentration on the ESR spectra of labelled membranes must next be investigated. It is desirable to perform successive probe titrations on a single membrane sample. The quantitative transfer of the probe to the membrane may be performed as described in *Materials and Methods.*

A common *in vitro* membrane labelling procedure utilizes bovine serum albumin (BSA) as a probe-transfering agent [23]. However, a careful probe titration experiment would be quite difficult with this method. Furthermore, Aoki and Foster [1] found that many albumin preparations, including the crystallized protein, contain protease contaminants active from pH 2.5 to 11.4. Several investigators have reported the effects of proteases on the spectral properties of spin-labelled membranes [18, 45, 70, 73].

C. The spectral parameters $T_{\parallel}$, $T_{\perp}$, ΔH, S, S', S'' and /or m derived from the above spectra should then be plotted as a function of probe concentration. If the "low" probe concentration range is identified from these plots, intrinsic membrane properties may be readily calculated. Membrane titration experiments performed with $I(m, n)$ probe samples synthesized and stored under different conditions will probably not yield identical results, since nitroxides may be oxidized or reduced easily [71].

D. It is sometimes possible to measure $T_{\parallel}$, but not $T_{\perp}$, in a $I(m, n)$-labelled membrane study. If titration experiments indicate probe/lipid ratios where $T_{\parallel}$ is independent of the probe concentration, then S'' may be used as a measure of membrane fluidity [Eq. (7)]. It must, however, be remembered that S'' is a function of both the motion of the probe and the polarity of the environment of the probe.

E. The above procedure could be modified to identify "low" probe concentrations for membrane systems which destroy the ESR signal of the spin label with time. Probe-mediated spectral perturbations could be monitored in such systems by initially loading the membrane with probe and continuously measuring ESR spectra. The decrease in probe concen-

tration could be estimated from a double integration of the ESR trace; the various spectral parameters could then be plotted as a function of decreasing concentration with time.

F. Care should be taken to (1) insure that the sample temperature is constant during the spectral measurements; (2) reproducibly position the ESR silica cell in the sample holder; and (3) maintain the microwave power at a fixed value which does not saturate the ESR signal or have a demonstrable sample heating effect.

II. The spectral alterations noted upon addition of an agent to a labelled membrane may be caused by changes in (1) the motion of the probe; (2) the polarity of the environment of the probe; and/or (3) probe-probe interactions. An otherwise ambiguous interpretation might be resolved by examining the functional relationship between agent-induced alterations in the hyperfine splittings and the probe/lipid ratio. If the magnitude of the spectral alteration is independent of probe concentration in the "low" range, then agent-induced changes in radical interactions are probably not involved.

This paper is dedicated to the late Mrs. Louise Holmes Copp of the California Metabolic Research Foundation.

Dr. Larry M. Gordon is a Postdoctoral Fellow of the San Diego County Heart Association, San Diego, California.

The authors wish to thank Dr. Arne N. Wick for the use of his facilities and for his encouragement, Judy A. Esgate for technical assistance, and Dr. Alfred Esser for reading the manuscript. The reviewers are acknowledged for their useful suggestions. This study was supported in part by U.S. Public Health Service Grant 5685-12 to Dr. Arne N. Wick.

Note Added in Proof

A $I(12,3)$-erythrocyte membrane study (Butterfield, D.A., Whisnant, C.C., Chesnut, D.B. 1976. *Biochim. Biophys. Acta* **426**:697) reported the following effects as the probe/lipid ratio increased from 1:83 to 1:8.3: (1) $T_{\perp}$, but not $T_{\parallel}$, increased; (2) the S [Eq. (8)] and m [Eq. (9)] values decreased by 4.4 and 2.1 ‰, respectively; (3) the high field peak height of the inner hyperfine doublet decreased below the baseline; and (4) a small "fluid" component appeared. These erythrocyte membrane results are in qualitative agreement with our own $I(12,3)$ titrations of liver and heart plasma membranes. Although the onset of high probe concentration "markers" in erythrocyte membranes apparently occurred at higher probe/lipid ratios than those noted for liver membranes, experiments were not performed at lower probe concentrations to unequivocally establish 1:83 as a low probe/erythrocyte membrane lipid ratio.

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