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SPIN LABELS: NEW BIOLOGICAL APPLICATIONS FOR ESR

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These new probes of biological structure and function can be used in systems that are opaque to visible and ultraviolet light. They are sensitive to the local environment, molecular orientation, and molecular motion of the spin-labeled molecule.

In the past, electron spin resonance (ESR) spectroscopy has been used in various fields of biological and biophysical research. The technique can be applied only to systems that contain some paramagnetic component, since the resonance requires the presence of unpaired electrons. Free-radical intermediates in enzyme reactions, as well as the more stable free radicals produced by ionizing radiation, have been studied by ESR. Most biological systems are basically diamagnetic, however, and require some perturbation to produce the free radicals necessary for ESR investigation. This absence of unpaired electrons has limited the versatility of the technique for biological research.

Recently, a new approach has been developed for introducing paramagnetic species into the normally diamagnetic biological system. Nitroxide free radicals are stable in aqueous and nonaqueous solutions for months or possibly years. Through ingenious construction of these nitroxide radicals--i.e., through appropriate choice of nitroxide--the various components of a biological system can often be spin-labeled quite specifically, either covalently or by noncovalent interactions. As there are no interfering signals from the unlabeled environment, the diamagnetic nature of organisms becomes an asset rather than a liability. Valuable information is contained in the ESR spectra of spin-labeled biological macromolecules.

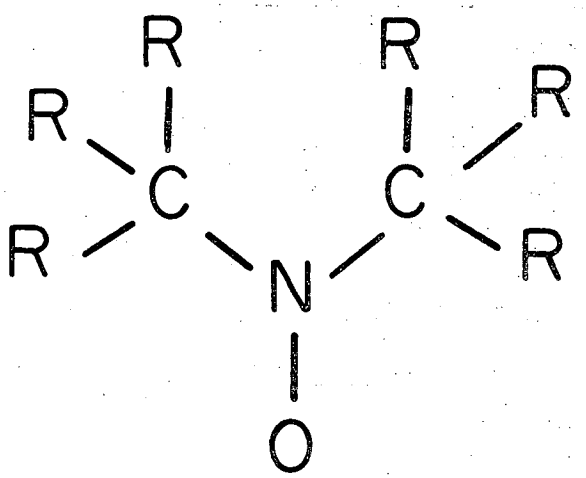
Analysis of Nitroxide ESR Spectra

In a strong magnetic field, an unpaired electron is oriented either parallel or antiparallel to the field direction. These two orientations, called spin states, correspond to two different energies for the unpaired electron, and spin resonance occurs when electrons are promoted from the lower energy level to the upper level. This requires electromagnetic energy, typically in the microwave region, which must be supplied by the ESR spectrometer. The condition for resonance is that the energy $h\nu$ of the microwave radiation be equal to the energy difference between the two electron spin states. This energy difference depends on the magnetic field strength H of the spectrometer and on a spectroscopic parameter, the g-value, which is determined by the electronic structure of the free radical. The familiar "resonance condition" for ESR is then

$$h\nu = g\beta H, \qquad (1)$$

where β is a constant, the Bohr magneton. Equipment requirements for ESR spectroscopy are an electromagnet, microwave apparatus, and sufficient electronic equipment for obtaining good sensitivity. Complete ESR spectrometers are available commercially from several sources. Those most commonly used display the ESR spectrum as the derivative of the microwave power absorption vs magnetic field strength.

From Eq. 1, a single absorption line would be expected, with its position in the magnetic field plot being determined by the g-value. The electronic structure of stable nitroxide free radicals of the general form



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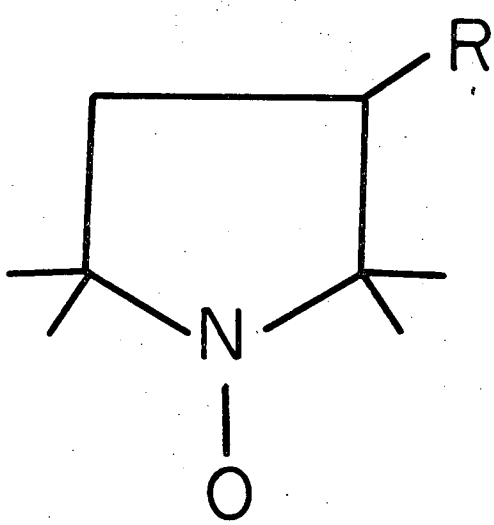
is such that the unpaired electron interacts with the nucleus of the nitrogen atom. This interaction, called hyperfine coupling, splits the absorption into three lines of equal intensity and equal spacing. The nitrogen hyperfine coupling, A^N, is another parameter (in addition to the g-value) that contains potentially valuable information. A third parameter, the width W of the absorption lines, depends on various environmental factors,

and in general is different for each of the three hyperfine components. Since all three lines are of equal intensity, a broad line has less amplitude h on a derivative absorption curve. Figure 1 shows the spectral parameters for a nitroxide in a somewhat viscous environment. The g-value is determined by the position of the center of the spectrum, which in this case is the central line, according to Eq. 1. With a suitable choice of probes and a knowledge of what factors influence the parameters g, A^N, and W, the researcher can design experiments with a versatility limited only by his imagination.

Choosing a Spin Label

Two classes of nitroxide spin labels are used most frequently as probes. Early spin-labeling studies employed almost exclusively the first class, which are the five- or six-membered cyclic nitroxides with structures I and II.



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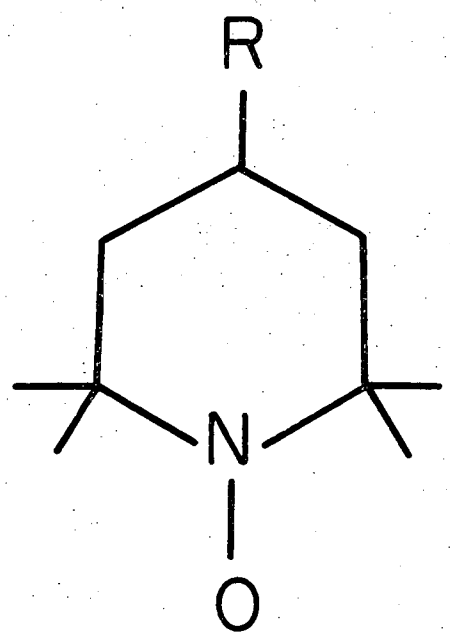

(II)

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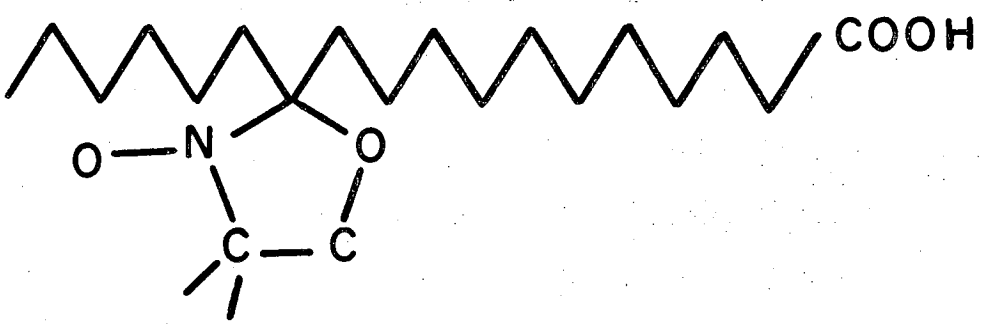
The functional group R is determined by the specific reaction required for labeling the molecule of interest. This group may be one that reacts chemically to provide a covalent bond to the macromolecule, or it may be one with weaker forces such as hydrogen bonding or hydrophobic interactions. An inherent disadvantage in probes of this type, for many studies, is that the linkage between the nitroxide moiety and the macromolecule allows some relative motion between the two. This introduces some ambiguity in correlating the motion of the macromolecule with that of the nitroxide group.

A more recent class of spin labels generally developed for attachment to ketone sites has a linkage that binds the nitroxide rigidly to the remainder of the molecule. An example of this class is the stearic acid derivative



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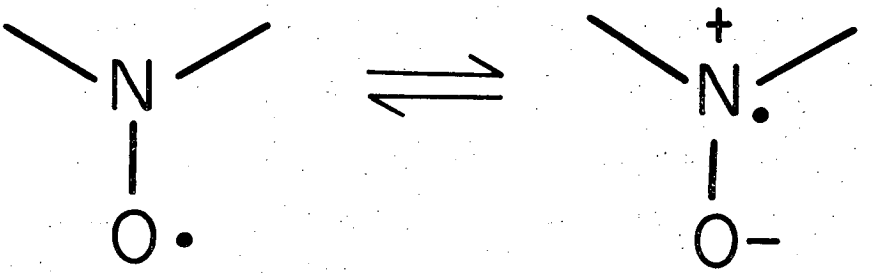
In this case, the mobility of the nitroxide more accurately reflects the mobility of the molecule as a whole.

Factors Affecting g and A^N

Both the g-value and the nitrogen hyperfine coupling depend on the orientation of the nitroxide free radical in the magnetic field of the spectrometer. If the molecules are tumbling very rapidly, however, the

spectrum obtained is simple and has average values for these parameters. These average values are called the isotropic g-value and the isotropic hyperfine coupling.

If the unpaired electron in a nitroxide radical were completely localized on the oxygen atom, there would be no isotropic coupling to the nitrogen nucleus. The observed coupling arises from the charged resonance structure



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where the unpaired electron is on the nitrogen atom. This charged structure is stabilized to a greater extent when the nitroxide is in a polar medium of high dielectric constant, such as water, than when it is in a nonpolar hydrocarbon environment. The result is that the same probe has a larger isotropic nitrogen coupling in a polar environment than in a nonpolar environment. There is also a solvent effect on the g-value, since this parameter is generally larger for an unpaired electron on oxygen than on nitrogen. Table 1 gives the isotropic values of g and A^N for nitroxide II(R = OH) in several different solvents. It is

apparent that A^N increases and g decreases with increasing dielectric constant of the solvent.

TABLE 1. Nitrogen hyperfine couplings (gauss) and g values for nitroxide II (R = OH) in solvents of different dielectric constant.

Solvent	Dielectric Constant	Hyperfine Coupling	g Value
n-Hexane	1.9	15.2	2.0061
Ethyl ether	4.3	15.3	2.0061
t-Butanol	10.9	16.0	2.0059
n-Butanol	17.8	16.1	2.0059
Ethanol	24.3	16.1	2.0059
Methanol	32.6	16.1	2.0059
Ethylene glycol	37.7	16.4	2.0058
Water	78.5	17.1	2.0056

In a biological system, this solvent effect can be used to determine the polarity of the region surrounding the spin label. Hubbell and McConnell[1] found that nitroxide II (R = H), diffused into several membrane systems, was located in two distinct regions of different polarity. The effect of polarity on g and A^N was such that the low-field and mid-field lines of the two spectra overlapped, but the high-field line was clearly resolved into two components. Both the polar and nonpolar regions were of low viscosity, in that the probe tumbled rather freely in both environments. The relative intensities of the two high-field components could be used as a measure of the partitioning of the probe between the two environments, and this was found to change with the addition of other molecules to the

system. It appears that polarity effects on g and A^N can be used to good advantage in heterogeneous systems.

Factors Affecting Line Widths

Oxygen Broadening

Line broadening by oxygen is often undesirable but in some cases may be used to the experimenter's advantage. Oxygen, being paramagnetic, can cause local variations in the net magnetic field surrounding the nitroxide, i.e., the field due to both the spectrometer and the magnetic dipole moment of oxygen. The result is a broadening of the spectral lines, since, at any time, different nitroxide molecules experience slightly different net magnetic fields. All three hyperfine lines are broadened equally by the presence of oxygen, in contrast to the effect of restricted motion on the line width. Figure 2 shows spectra of nitroxide II (R = OH) in hexane and water, with and without oxygen present. The broadening effect is quite pronounced in the hydrocarbon solution but is not noticeable in water. This may be due to a much greater clustering of the polar oxygen molecules about the polar nitroxide molecule in a nonpolar solvent than in water.

Although no biological studies have been reported that make use of oxygen broadening, it seems that this effect might be used to detect local regions of high oxygen concentration in nonhomogeneous systems. Such determinations might prove more useful than measurements of the average oxygen concentration in cases in which there are large local differences in oxygen concentration.

Exchange Broadening

Like oxygen broadening, this effect can be both detrimental and useful. Exchange broadening occurs when the nitroxide concentration becomes so high that significant electron exchange occurs between adjacent molecules. In most cases, especially when line broadening due to restricted molecular motion is of interest, it is best to keep the spin-label concentration low enough to avoid exchange interaction. In a homogeneous system, safe concentrations are about 10^{-3} M or less.

In nonhomogeneous systems, exchange broadening often reflects very high local concentrations of spin label in a system whose average nitroxide concentration is quite low. Figure 3a-e shows spectra of a hydrocarbon probe in a 0.5% aqueous solution of sodium dodecylsulfate. The sodium dodecylsulfate at this concentrations forms micelles containing regions that are quite hydrophobic in nature. The hydrocarbon probe is insoluble in water, but dissolves readily in the local micellar hydrocarbon region.

In Fig. 3a, with only 1.1 probe per micelle, the spectrum shows no exchange broadening. The increased width of the high-field line is due to the restricted motion of the probe within the micelle. With increasing nitroxide concentration, the lines all broaden and finally coalesce into a single, unresolved line. The average concentration, 3×10^{-2} M, at which this occurs is much less than would be required if the system were homogeneous. For comparison, Fig. 3f shows the spectrum of a 10^{-2} M solution of nitroxide II (R = OH) in water. There is little, if any, exchange broadening evident here, even though the average concentration is about the same as for Fig. 3d. It is clear

that exchange broadening is a sensitive detector for very high local radical concentrations.

Molecular Motion

Perhaps the most useful information obtained from spin-label experiments to date has come from the effect of restricted molecular motion on the line widths of the nitroxide spectra. A nitroxide radical which is freely tumbling in solution, with a rotational correlation time (τ_c) less than about 10^{-10} sec, exhibits only the isotropic values of g and A^N, since the anisotropic dipolar contributions are averaged out by the rapid motion. In this case, the three nitrogen hyperfine lines have approximately the same line widths, and a symmetric spectrum is observed. Restriction of the molecular motion, such as occurs in a viscous solution, has the effect of introducing contributions from the anisotropic parts of g and A^N into the ESR spectrum. The pattern becomes somewhat asymmetric, with the high-field line having a greater width than the other two lines. Equations have been derived that relate the rotational correlation time to the ESR line widths. Application of these equations requires a knowledge of both the isotropic and anisotropic contributions to g and A^N, these parameters generally being determined from studies on single crystals. Fortunately, most nitroxides have quite similar values for the spectral parameters, and these have been measured for a few spin labels.

The appropriate line-broadening equations,

$$\tau_c^{(1)} = \frac{15\pi\sqrt{3}\,W_0}{8b\Delta\gamma H}\left[\frac{W_{-1}}{W_0} - \frac{W_1}{W_0}\right],$$

$$\tau_c^{(2)} = \frac{4\pi\sqrt{3}\, W_0}{b^2}\left[\frac{W_{-1}}{W_0} + \frac{W_1}{W_0} - 2\right],$$

provide two ways to calculate τ_c, and these give an internal check for consistency. The quantities b and $\Delta\gamma$ are related to the anisotropies in A^N and b respectively. Using spectral parameters of Griffith et al.[2], and with H = 3400 gauss, we can reduce these to

$$\tau_c^{(1)} = 6.5\times10^{-10}\, W_0\left[\left(\frac{h_0}{h_{-1}}\right)^{1/2} - \left(\frac{h_0}{h_1}\right)^{1/2}\right],$$

$$\tau_c^{(2)} = 6.5\times10^{-10}\, W_0\left[\left(\frac{h_0}{h_{-1}}\right)^{1/2} + \left(\frac{h_0}{h_1}\right)^{1/2} - 2\right].$$

Here, the ratios W_{-1}/W_0 and W_1/W_0 have been replaced by $(h_0/h_{-1})^{1/2}$ and $(h_0/h_1)^{1/2}$, where h is the line height of the first derivative spectrum. The line-height ratios are usually more easily and accurately measurable than the line widths, and the substitution assumes only that the lines are Lorentzian in shape. Numerically, the line width W_0 is in gauss.

This method for monitoring molecular motion may be applied for measuring rotational correlation times from about 10^{-10} to 10^{-8} sec. For more restricted motion, the line widths become so great that the individual lines overlap, and it becomes difficult to make accurate line-width measurements. Spectra observed for motion in the range of 10^{-8} sec to the completely immobilized state are in general quite complicated to analyze. For the more rigid case, such as those of Fig. 4, the asymmetric spectrum has three prominent peaks. The separation between the outer components is approximately twice the isotropic coupling A^N. Although all spectra similar to those in Fig. 4 are often referred to as "rigid glass spectra," it is obvious that different degrees of rigidity may be seen.

To somewhat quantitate estimates of molecular mobility in the range where the line-broadening equations cannot be applied, an empirical relation based on suitable spectral parameters such as the two heights a and b of Fig. 4 may be used. Stokes' law relates the rotational correlation time of a spherical object, of radius r, to the viscosity η of the medium, by

$$\tau_c = \frac{4\pi\eta r^3}{3kT} ,$$

where k is Boltzmann's constant and T the absolute temperature. Values of η at different temperatures for various solvents are known, and it would be expected that the temperature effect on viscosity would be reflected by the mobility of the dissolved nitroxide. In the range -5° to -45°C, the viscosity of glycerol changes by a factor of approximately 750 (Fig. 5). The ratio a/b for nitroxide II (R = OH) in the glycerol solution varies smoothly in this temperature range, and Fig. 5 shows the relation between a/b and the viscosity. Although there is no simple way of correlating τ_c with spectral parameters such as a/b, the point to be made is that the ratio a/b reflects quantitatively changes in mobility, perhaps by a factor of several hundred, in a range where the line-broadening equations are inappropriate and where all spectra are often referred to as representing complete lack of motion.

Difficulty may be encountered in attempting to relate τ_c as determined by Stokes' equation to that calculated from the line-broadening equations, even for a molecularly homogeneous system. The problem apparently arises from a vast discrepancy between the bulk viscosity of a solution and the local viscosity encountered by the nitroxide free radical.

As an example, the spectra of nitroxide II (R = OH) in octadecane at 40° and 20°C are shown in Fig. 6a, b. The bulk viscosity for these two cases is different by several orders of magnitude, since at 40°C octadecane is a liquid, whereas at 20°C it is a solid. On the other hand, the two spectra are very similar, which shows that the local viscosity in the vicinity of the probe changes only slightly. The effect is not limited to octadecane, as can be seen in Fig. 6c, nor is it limited to the particular nitroxide used in this example. For biological processes, the local viscosity, at the molecular level, may well be more significant than bulk viscosity as measured for isolated components, and the nitroxide spin labels should serve as excellent sensors for localized motion in an otherwise seemingly rigid environment.

Complex Spectra

In complex systems, a simple three-line spectrum does not always appear. The resulting spectra may be complex and the reasons numerous; some of the less complex examples are discussed.

In some heterogeneous systems a probe may be in two states of immobilization; therefore, three sharp lines superimposed on a broadened spectrum are observed.

Other heterogeneous systems, such as a phospholipid dispersion in water, partition certain small nitroxides between the hydrocarbon and the aqueous phases. In the example shown (Fig. 7), two low-field lines and two high-field lines appear. This complex spectrum is caused by alteration of A^N by the probe's local environment. At room temperature (Fig. 7a) the high-field hydrocarbon line is broadened; at higher temperatures (Fig. 7b), the hydrocarbon lines are noticeably sharpened. Further-

more, the partition coefficient of the nitroxide is also altered at elevated temperatures, so that a greater proportion now resides in the hydrocarbon phase. Figure 7c shows the effect of Vitamin C, a water-soluble reducing agent, on the mixed spectrum. Part of the signal in the aqueous phase has been destroyed but that in the hydrocarbon is undisturbed. Complex spectra may occur frequently in biological systems, and the experimenter must go to great lengths to selectively destroy or mathematically simplify the signal.

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2. O. H. Griffith, D. W. Cornell, and H. M. McConnell, J. Chem. Phys. 43, 2909 (1965).

The interested reader should consult recent reviews by Hamilton and McConnell in Structural Chemistry and Molecular Biology, A. Rich and N. Davidson, eds. (W. H. Freeman and Company, San Francisco, 1968), and Griffith and Waggonner, Accounts Chem. Res. 2, 17 (1969), where many references to the current spin-label literature are given. The chemistry of nitroxides is described by Forrestor, Hay, and Thomson in Organic Chemistry of Stable Free Radicals (Academic Press, Inc., New York, 1968).

FIGURE LEGENDS

Fig. 1. First derivative ESR spectrum of a nitroxide free radical.

Fig. 2. Effect of oxygen on line widths in hexane and water.

Fig. 3. Exchange broadening by high local probe concentrations in micelles of sodium dodecylsulfate.

Fig. 4. Different degrees of rigidity in extremely viscous environments.

Fig. 5. Relation of viscosity to temperature and the ratio a/b for nitroxide II (R = OH) in glycerol.

Fig. 6. Relatively free motion of a nitroxide in "solid" environments.

Fig. 7. Complex spectra from a nitroxide partitioned between aqueous and hydrocarbon environments.

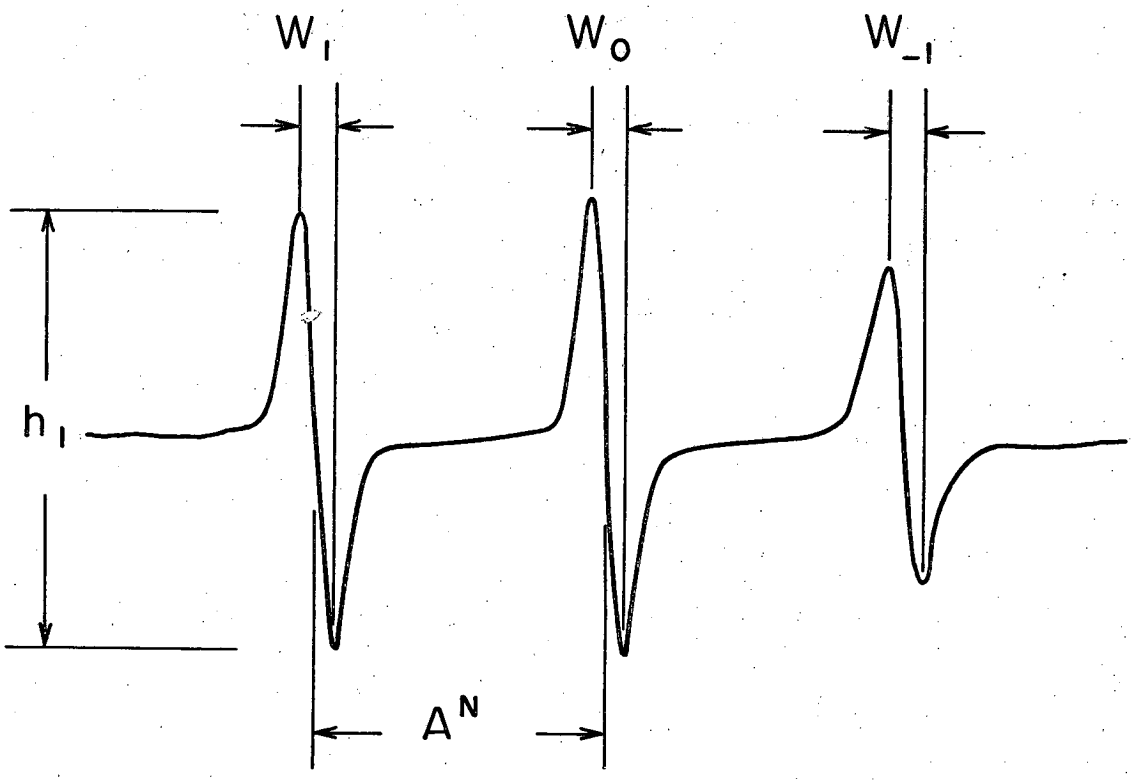

Fig. 1

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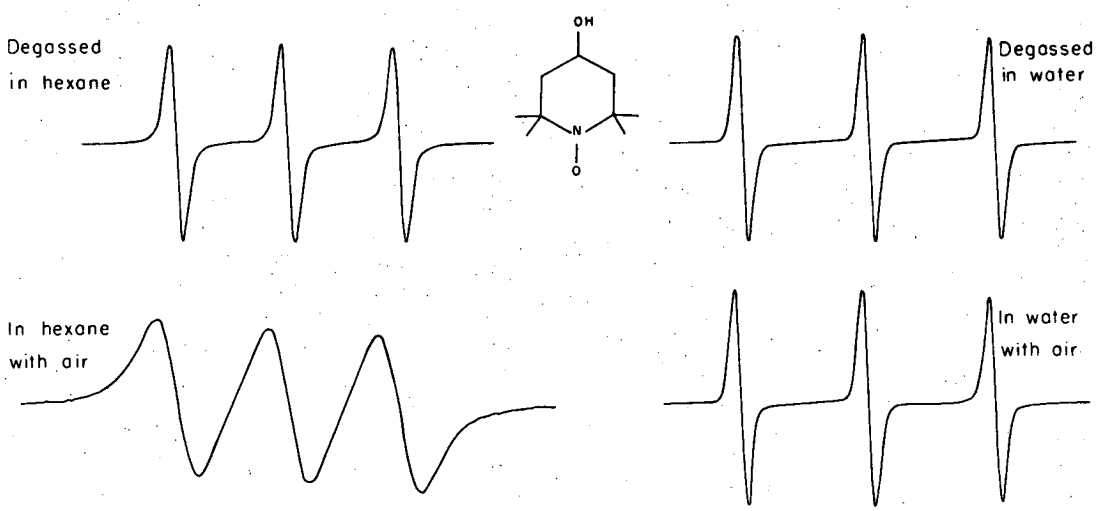
Degassed
in hexane
OH
N
O
Degassed
in water
In hexane
with air
In water
with air
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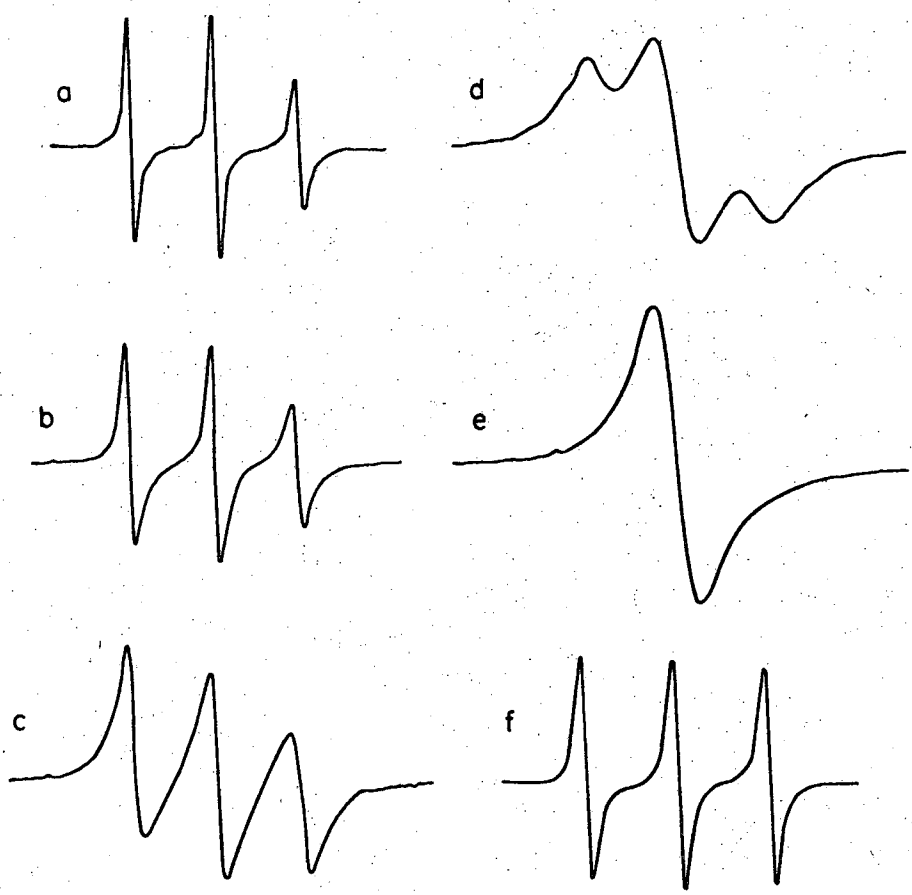
Fig. 2



Concentration (mM)		probes / micelle
a	0.3	1.1
b	0.9	3.3
c	3	11
d	9	33
e	30	110
f	10	

Fig. 3

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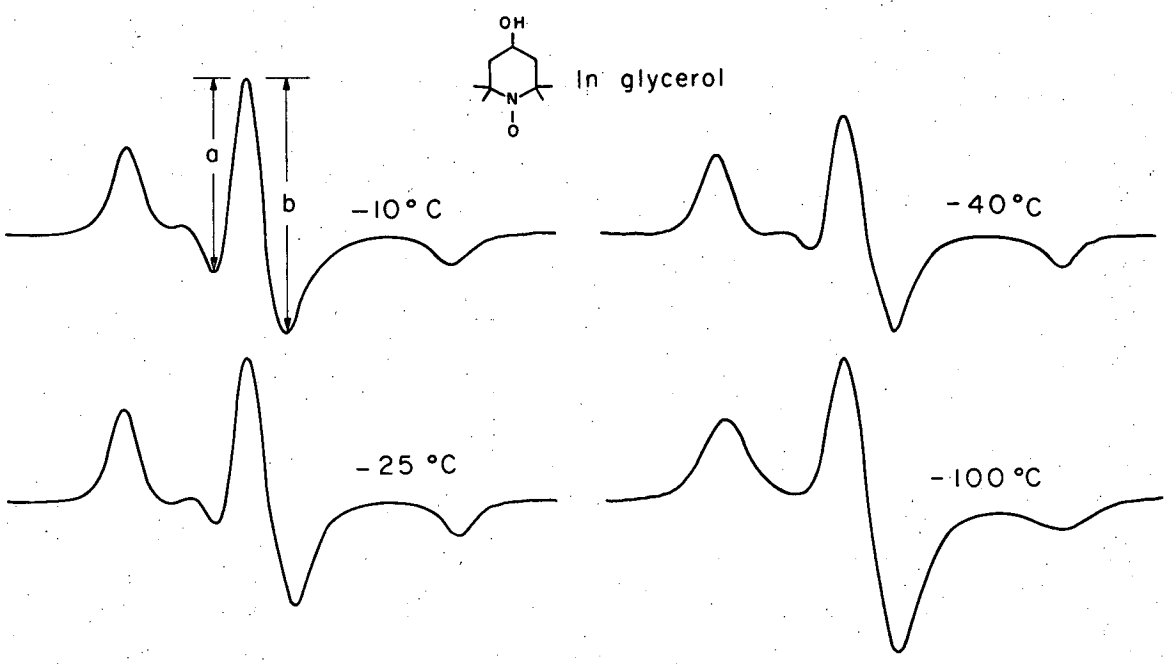

Fig. 4

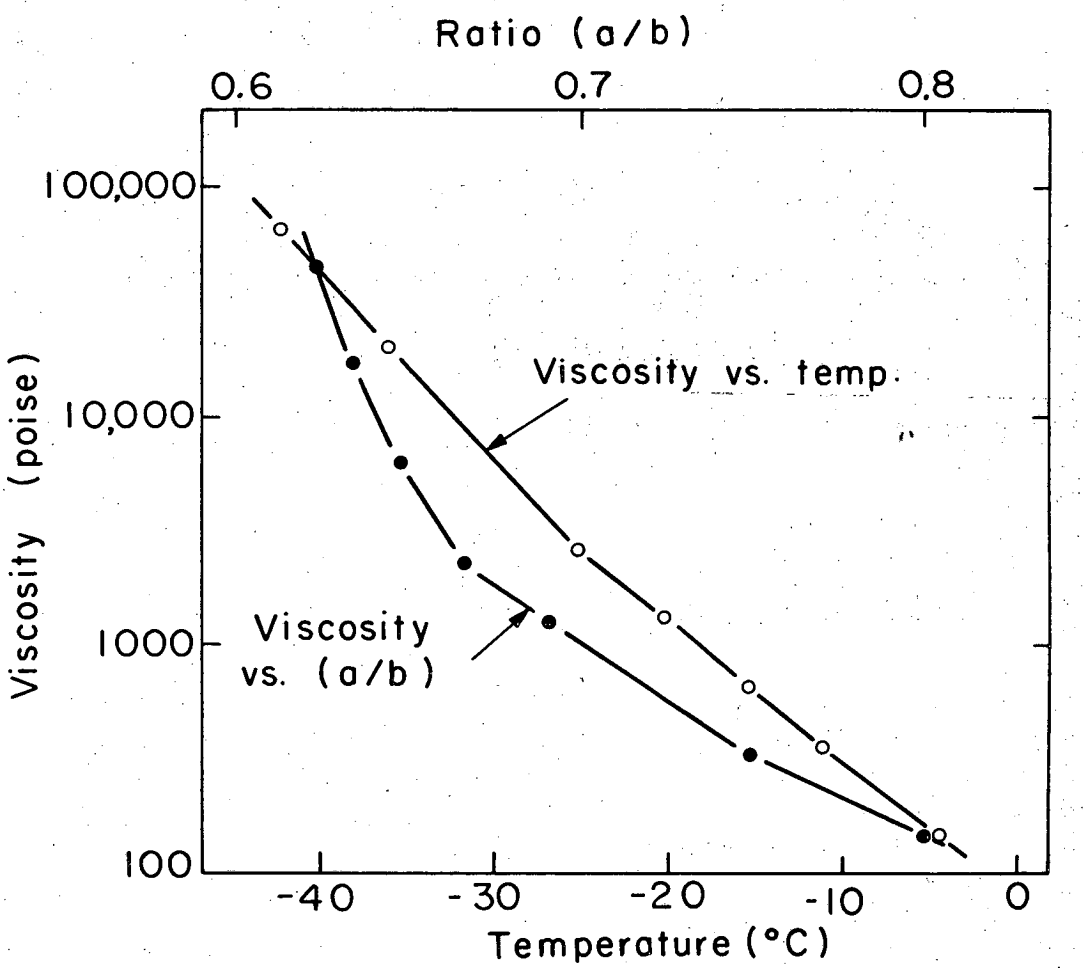

Fig. 5

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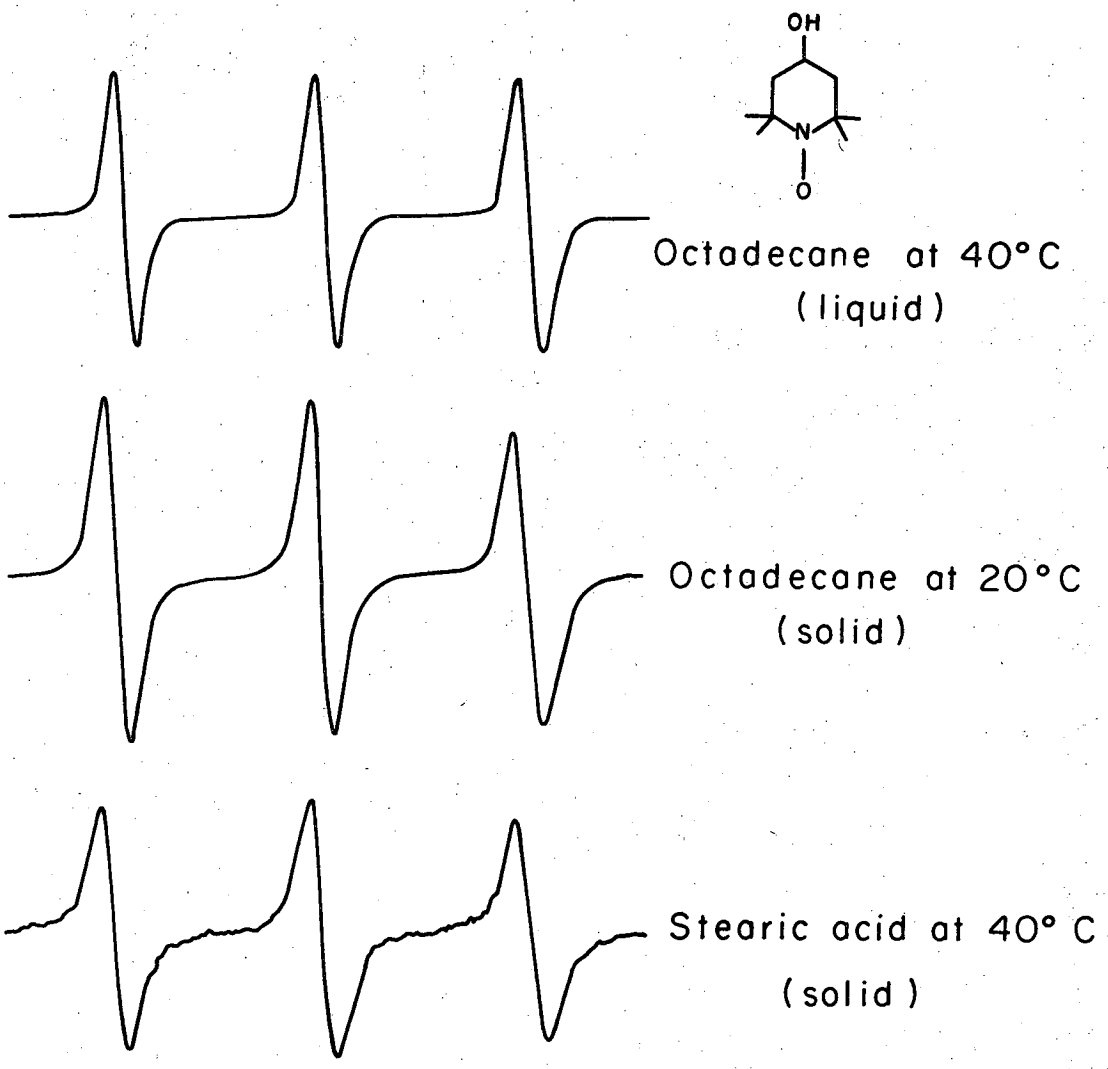

Fig. 6

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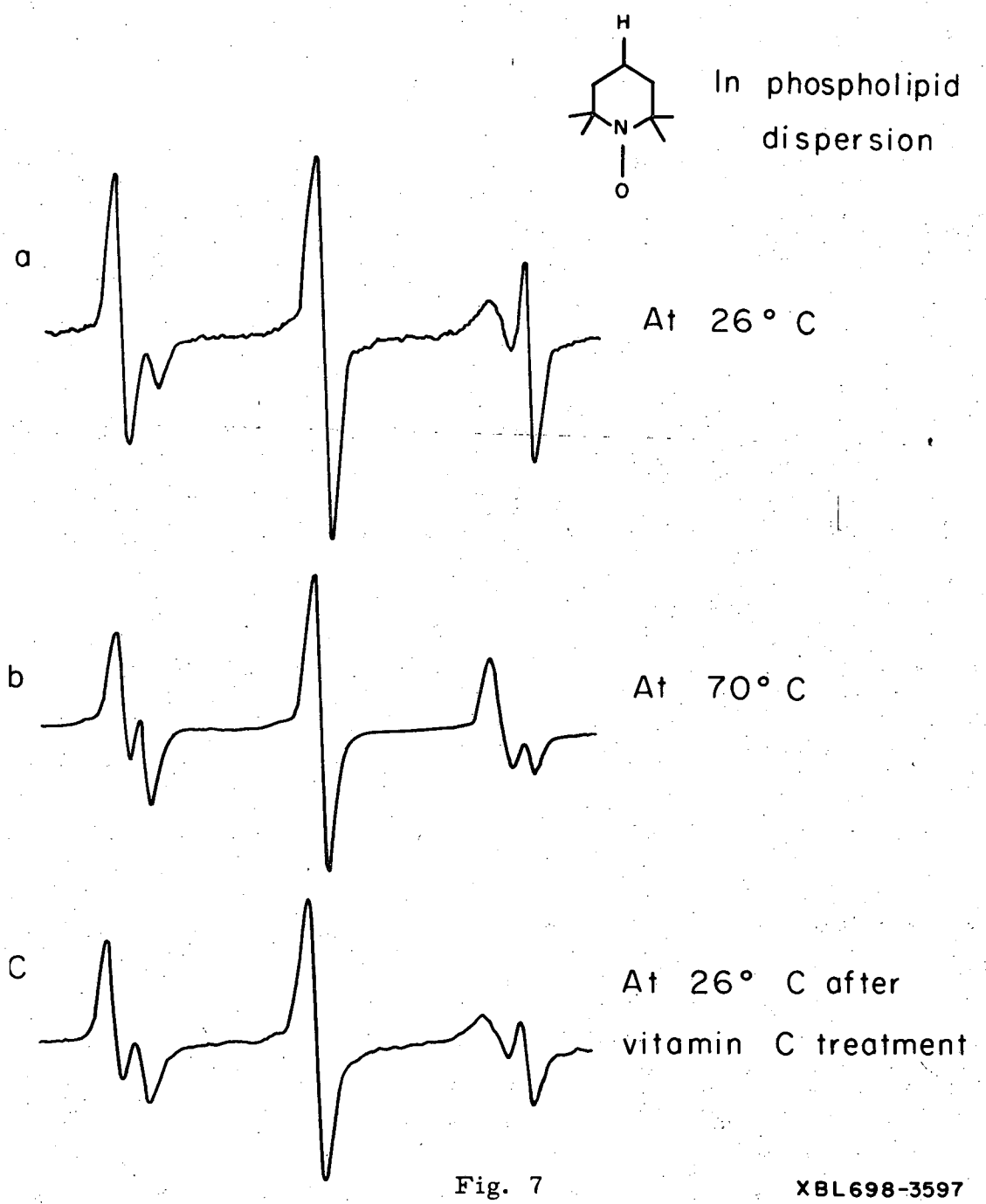

Fig. 7

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